THE ROLE OF SEMIOCHEMICALS IN THE BEHAVIOR OF THE HORN FLY, *Haematobia irritans* (L.), (DIPTERA: MUSCIDAE)

By

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To Bryce and Adam B.

As long as the moon rises,
As long as the grass grows green,
As long as the river flows,
We will be friends,
We will live in peace.

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The courtship behavior of mature, well-nourished horn fly, Haematobia irritans (L.), males and females was similar to that of other muscids; however, unlike other muscids during one element of the courtship sequence, the male rubs the female's head with its protarsi. The duration of courtship and copulation was variable among pairs. Virgin males courted tethered, virgin females with greater frequency than they courted tethered, virgin males. Virgin males could readily differentiate females from males and court females regardless of whether the horn flies were live, dead, winged, or wingless.

Preliminary bioassays on the horn fly suggested that a mating stimulant pheromone was involved in horn fly courtship behavior. Virgin males readily courted live or dead females but rarely made mating strikes upon live males, dead males, or females thoroughly washed with hexane. Bioassays utilizing the response of virgin males to treated, virgin male horn flies indicated that female cuticular hydrocarbons were responsible for inducing male courtship behavior. Specifically, the female paraffin

and monoolefin fractions were biologically active when bioassayed alone or in combination. Three synthetic monoolefins previously shown to be the major components in the female monoolefin fraction were biologically active in bioassays. The compounds (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene were each active, but greater male courtship behavior was observed when these 3 compounds were bioassayed in combination.

Florida Laboratory Strain (F) males and females and Florida Wild Strain (W) (field-collected) males and females had similar analytical gas chromatograms for total paraffins. The F and W females had relatively greater quantities of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene than did F and W males; F and W males had relatively greater quantities of (Z)-9-tricosene. Bioassays utilizing the response of virgin males to treated, virgin male horn flies previously washed with hexane indicated that (Z)-9-tricosene did not attenuate the courtship behavior of males toward dead females. Additionally, these bioassays demonstrated that with increasing doses of a 1:1:1 combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene, the frequency of successive elements of the courtship hierarchy of the male was increased.

The combination of the 3 cuticular monoolefins previously demonstrated to be active as a mating stimulant for male horn flies was tested as an attractant to horn flies in laboratory olfactometer studies and in simulated field trials conducted in outdoor screened enclosures. The combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene was attractive to virgin male horn flies in olfactometer trials; however, in a simulated field trial, the combination was not attractive to either sex at the dose tested.

CHAPTER 1

INTRODUCTION

Concomitant with the increased use of synthetic pesticides after World War II was the decline in use of preventive practices for pest control and the waning of interest in biological control studies. Investigation of the long-term effects of pesticide use in the environment was minimal. As resistance to insecticides developed, public awareness of the environmental impact of pesticide usage increased, and anxiety about the acute and chronic effects of pesticides on man emerged, pest control strategies came under scrutiny (Brand et al., 1979).

The concept of integrated pest management began to form as a holistic approach to pest control utilizing alternative control methods while maintaining pesticides as viable tools where appropriate. Among alternative control methods, behavior modifying chemicals including pheromones have been investigated more thoroughly as the chemical basis of insect behavior has become firmly established. Practical applications of these chemicals include: trapping difficult to obtain species, trapping to monitor or survey for specific pest species, luring insects to areas treated with pathogens or pesticides, mass trapping to suppress pest populations, and disruption of insect communication (Brand et al., 1979).

After the early success of the Southeastern Screwworm Eradiation Program, Knipling (1972) suggested that eradication of the horn fly was

feasible because this ectoparasite remained in close contact with cattle and was, therefore, accessible to control. He proposed an integrated control program involving insecticide treatments to reduce low population numbers in the beginning of the horn fly season, followed by release of sterile males to eliminate the remaining horn fly population. Field trials with this general strategy have been handicapped by difficulties in mass rearing the required numbers of horn flies and by the abnormal behavior of released flies (Eschle et al., 1973; Graham and Hourrigan, 1977). Chambers (1977) emphasized that the production and perception of pheromones may be important in the reproduction of many species; changes in the mating behavior of mass reared insects may be indicative of a lack of adaptiveness critical to reproductive success. Control of the quality of mass reared insects requires an understanding of the behavior of the insect paramount to their success in the field. Laboratory evaluation of this behavior is needed to insure competitiveness of mass reared individuals with their wild counterparts.

Presently, insect chemical communication is viewed as being more complex than was originally envisioned. Ultimate use of this communication in pest management programs requires additional study of chemically induced behavior under defined contexts and conditions (Birch, 1978).

The purpose of this investigation was threefold: (1) to examine and describe the courtship behavior of the horn fly in the laboratory; (2) to determine if cuticular lipids were involved as semiochemicals (Nordlund and Lewis, 1976) in horn fly courtship; and (3) to investigate candidate pheromones involved in horn fly courtship as possible

attractants or compounds which might influence the movement of horn flies among animals in the field.

This dissertation consists of a literature review of the horn fly, the sex pheromones of the Muscidae, and the courtship behavior of the Muscidae as well as four chapters containing experimental work, written in manuscript form, to be submitted for publication.

CHAPTER 2

LITERATURE REVIEW

The Horn Fly

History and Distribution

The horn fly, Haematobia irritans (L.), is an Old World species and an obligate parasite of cattle as an adult. Although adult horn fly populations on animals in Europe are usually low, the horn fly rapidly became a serious pest of livestock in the United States after its introduction with cattle imported into Philadelphia from Europe in 1885 or 1886 (Marlatt, 1910; Bruce, 1964; Graham and Hourrigan, 1977). First recognized in 1887 by Riley (1889), the horn fly rapidly spread to Michigan by 1892 (Davis, 1893), to California by 1893 (Bruce, 1964), and to Hawaii by 1897 (James and Harwood, 1969). By 1900, horn flies were reported in most of the United States, Canada, and Puerto Rico (Hargett and Goulding, 1962). Currently, the horn fly is cosmopolitan in the New World with a general distribution from Venezuela to Canada (Hargett and Goulding, 1962; Stone et al., 1965; Graham and Hourrigan, 1977).

Bionomics

Mating. On the host, horn flies normally mate within 3 days of eclosion with egg deposition beginning one day later (Bruce, 1942 and

1964; Harris et al., 1968; Schmidt, 1972). In the laboratory, females begin to mate as much as 24 hours later than when held on the host (Harris et al., 1968). The female horn fly appears to be monogamous, but males can inseminate an average of 4.6 females (Harris et al., 1968).

Oviposition. Although the specific stimuli which induce egg laying are not known (McClintock and Depner, 1954), adult female horn flies leave their host to oviposit in freshly passed cattle droppings or on the grass or soil beneath the dropping (McClintock and Depner, 1954; Bruce, 1942 and 1964; Harris, 1962; Sanders and Dobson, 1969). Droppings older than 10 minutes are unattractive unless the crust is broken (Bruce, 1964). Greer and Butler (1973) demonstrated that horn fly larvae could develop in cattle, bison, sheep, and horse manure; however, larvae are believed to develop only in cattle manure in nature (Depner, 1961; Bruce, 1964). According to Bruce (1964), the female produces 24 eggs in each of 15 batches during her lifetime. By about as early as the third day after emergence, the female can spend up to 10 minutes depositing 1-14 eggs before returning to a host. Females are equally as active in egg laying during the day as during the night (Sanders and Dobson, 1969; Kunz et al., 1970).

Development. Schmidt et al. (1972) noted a higher percent egg hatch in more densely populated laboratory cages. In the field in summer, eggs hatch within 16-24 hours and the newly emerged larvae crawl into cracks and crevices in the manure. With drying of the manure, larvae migrate to more moist portions of the dropping (Bruce, 1942 and 1964; McClintock and Depner, 1954). Within a 4 day period, the larvae develop through 3 instars. The first instar, which is approximately 1.5 mm long by 0.25 mm wide, has no anterior spiracles but has heavily

pigmented knob-like posterior spiracles. At about 10.25 hours, molting occurs to the slightly larger second instar which has anterior spiracles with 4-6 branches. About 18-25 hours from the first molt, molting occurs to the third instar which is approximately 6.5 mm long and 1.0 mm wide and more highly pigmented than the first 2 instars (Bruce, 1964).

About 92.5 hours from hatch, larvae migrate to the underside of the dropping or into the soil dependent on the relative moisture content of the soil and the dropping for pupation (Bruce, 1964; Escher, 1977). The pupal stage lasts about 5.5 days under field conditions. Adults attempt to fly and locate a host about 1 hour after eclosion (Bruce, 1964).

Kunz et al. (1970) reported the peak emergence of adults in central Texas to range from 9-10 days after oviposition during the summer to 14-21 days after oviposition in September. Peak emergence varied by 3-4 days depending on whether the dropping was in the sun or shade. Fewer horn flies emerged from droppings inhabited by other insects than from droppings with low numbers of competing insects (Blume et al., 1970). Droppings artificially covered within 5 minutes after deposition to eliminate competition from other insects averaged 66.5 emerging flies per dropping while uncovered droppings averaged 6.6 emerging flies (Kunz et al., 1970). During the summer months in Florida, Wilkerson (1974) and Greer (1975) reported an average of 8-19 emerging adults per dropping.

Temperature and moisture greatly influence egg hatch and immature development. Melvin and Beck (1931) and Melvin (1934) reported that egg hatch occurred at 11.33 hours at 34.4°C and at 19 hours at 25°C with the total developmental time from egg to the adult of 9.9 days at 30°C.

The egg to egg life cycle has been reported to vary from 9-12 days in the field (Melvin and Beck, 1931; Bruce, 1942 and 1964; Hargett and Goulding, 1962) to a maximum of 32 days in the laboratory (Depner, 1962). Recently, Wilkerson (1974) reported developmental times from egg hatch to adult as follows: 8 days at 35.6°C; 8 days at 31.3°C; 9 days at 26.7°C; 11 days at 22.2°C; and 16 days at 17.8°C.

In both the field and the laboratory, the eclosion of horn flies follows a circadian rhythm with variation influenced by temperature (Harris et al., 1971; Hoelscher and Combs, 1971b). Females emerge as much as 24 hours earlier than males (McClintock and Depner, 1954; Harris et al., 1971; Hoelscher and Combs, 1971b). Glaser (1924) and Mohr (1943) have reported sex ratios of 1:1 to 1:1.35.

Feeding. Both sexes are obligate blood feeders as adults. Although early workers cited by Bruce (1942 and 1964) reported that horn flies fed two or more times a day especially at dawn and dusk, later investigations indicated that horn flies feed intermittently throughout the day especially when interrupted by host movement. Utilizing an electronic recording instrument, Harris and Miller (1969) established that females feed an average of 12 times per day with feedings distributed evenly throughout the day. Each feeding lasted an average of 1.2 minutes; cumulatively, 14.3 minutes per day were occupied in feeding. Harris and Frazar (1970) observed that males consumed only two-thirds as much blood as females.

Longevity and seasonal occurrence. Estimates of adult horn fly longevity range from 28 days (McClintock and Depner, 1954) to 6-8 weeks (Bruce, 1964); each estimate is highly influenced by ecological conditions (Bruce, 1964). In the laboratory, Schmidt et al. (1972) found

that greater adult survival occurred in less densely populated cages with the optimum density from 0.6-9.5 cm^3 per fly.

Bruce (1942 and 1964) hypothesized that temperature limits the presence or absence of horn fly populations while moisture determines their abundance; warm, moist weather is favorable for horn fly longevity while hot, dry weather and periods of low temperature are unfavorable. Morgan (1964) emphasized that the micro-climate of the hair coat mantle is equally important as is the macro-climate. In many areas of the United States, adults are found continuously on cattle from spring until late fall with possible population fluctuations throughout that period (Wright, 1970). In Florida, horn flies are found on the host year round; winter populations may average 15 flies per animal while summer populations are as high as 1000 flies per animal (Butler, 1975).

In the temperate and cooler regions, horn flies diapause in winter as either third instar larvae or pupae (Depner, 1962; Hoelscher et al., 1967; Kunz et al., 1972; Hoelscher and Combs, 1971a). In the tropics and subtropics, horn fly development occurs throughout the year (Hoelscher et al., 1967). In central Florida diapause in the horn fly has not been observed (Butler, 1975). Diapause in horn flies is believed to occur as the combined result of the changing photoperiod to which adults are exposed and the temperature which the immatures experience (Depner, 1961 and 1962). Depner (1962) hypothesized that the host may transmit a "UV" factor to the horn fly which in turn transmits this factor to its offspring predisposing the pupae to diapause.

Host preference. Although adult horn flies essentially spend their entire life upon cattle, other hosts such as sheep, goats, horses, mules, dogs, deer, and humans are occasionally attacked (Bruce, 1942

and 1964) especially if a bovine host is not present (Graham and Hourrigan, 1977). Horn flies are generally more numerous on black or dark-colored animals than on light colored animals (Bruce, 1964) and tend to rest on the dark areas of bi-colored animals (Burns et al., 1962; Franks et al., 1964). Morgan (1964) hypothesized that these differences in attraction are due to the flies seeking certain micro-environmental temperature and humidity requirements. While each cow in a herd can carry several hundred to 4,000 adult flies (Graham and Hourrigan, 1977) and occasionally 10,000 flies (Bruce, 1942), bulls are preferred with populations as high as 20,000 per animal (McClintock and Depner, 1954; Pfadt, 1962). Bimonthly injection of 250 mg of testosterone propionate increased the attractiveness of steers, but weekly injection of the same dose decreased attractiveness (Dobson et al., 1970). Short-haired breeds or individuals are preferred hosts (McClintock and Depner, 1954; Bruce, 1964), while Brahman cattle are less attractive than European breeds (Tugwell et al., 1969).

Flight Behavior

Dispersal. Although early workers reported that horn flies remained on the host continuously with the exception of the ovipositing female (Bruce, 1938, 1942, and 1964; McClintock and Depner, 1954) and those flies which were thought to be dislodged from the host at night and unable to locate a new host (Eddy et al., 1962; Hargett and Goulding, 1962; Bruce, 1964), current investigations indicate that horn flies can disperse over great distances from the host. Horn flies were captured up to 8.1 km from release points by Eddy et al. (1962) while Tugwell et al. (1966) found that adults could travel up to 14,100 m in

4 hours; females were found to move more than males while only 27 of 731 flies trapped on a pasture with 37 cows had been previously associated with those hosts (Tugwell et al., 1966). Greatest flight activity occurred in the morning. On the other hand, Hoelscher et al. (1968) found that fly movement was primarily nocturnal; movement of flies between animals penned 91.4 m apart was reported. Studies by Kinzer and Reeves (1974) indicated the following: 1) flies released in the early morning and evening were more successful in locating a host than those released during the day; 2) marked horn flies were recaptured in 10 hours from hosts located 11.8 km from the release point; 3) 68-77% of the flies on one host left that animal within 11 hours; and 4) males and females dispersed in equal numbers. Kinzer and Reeves (1974) hypothesized that males and parous or nulliparous females have a strong tendency to transfer between hosts. Investigation by Janes et al. (1968), Hayes et al. (1972), and Wilkerson (1974) indicate that a substantial number of flies do migrate between herds of cattle.

Laboratory studies by Sauerman (unpublished data) using flight mills demonstrated that recently emerged horn fly females can migrate up to 3.9 km on energy reserves from the larval stage; adult flies maintained on blood in the laboratory can fly more than 14.5 km. Although the necessity for repeated blood feeding of the horn fly may explain the tendency of the horn fly to seek a host (McClintock and Depner, 1954; Harris and Miller, 1969), it does not adequately explain to several investigators the strong tendency that horn flies have to transfer between hosts (Kinzer and Reeves, 1974; Hoelscher et al., 1968).

Orientation to a host. After conducting a series of experiments on the visual and olfactory responses of 2-3 day old horn flies collected from the host and recently emerged, honey-fed horn flies, Hargett (1962) and Hargett and Goulding (1962) concluded that vision was the most important factor in aiding the horn fly in finding a host after emergence. In addition, they found that horn flies: 1) responded greatest to light filtered at 550 mμ and shorter wavelengths; 2) were attracted to areas of contrast; 3) were attracted to bovine hair and extracts made from washing bovine hair; 4) probed in response to heat; 5) were negatively geotactic; and 6) showed a lack of humidity preference. They hypothesized that recently eclosed flies utilize light stimuli and negative geotaxis to begin flight. Areas of contrast in the fly's visual field may override light stimuli; when a specific host comes into view, vision directs the fly toward it. Kinzer and Reeves (1974) suggested that horn fly dispersal and location of hosts over distance were random in regard to wind direction, but directional in regard to temperature, wind velocity, and humidity.

Kinzer et al. (1970) reported slight but statistically significant attraction in laboratory olfactometer trials to cow odor and emanations from a human arm. In other olfactometer studies, Mackley (1977) demonstrated slight attraction of both males and females to the hydrocarbon fraction of female cuticular lipids. Kinzer et al. (1978) developed field olfactometers to measure the attractiveness of selected stimuli to host seeking horn flies. Temperature and CO_2 were the primary factors found to affect horn fly orientation.

Economic Damage

Direct damage. Stress from fly annoyance and blood loss, which occur when horn fly populations are above economic thresholds, causes significant loss in weight and milk production in cattle (Bruce, 1942 and 1964; McClintock and Depner, 1954; Hoelscher and Combs, 1971a). The preferred feeding sites on animals with heavy infestations may develop into open sores which are susceptible to screw-worm attack as well as invasion by other parasites or diseases (Bruce, 1942 and 1964). The daily consumption of 500 flies is about 7 ml of blood (Harris and Frazar, 1970); extremely high horn fly populations can cause blood loss which alone would reduce animal productivity (Butler, 1975). Reductions in calf production have been reported (McClintock and Depner, 1954). A one-fourth to one-half reduction in milk production in dairy herds not protected from biting flies was demonstrated by Granett and Hansens (1956).

Weight losses to cattle due to horn fly damage have been evaluated by comparing the weight change in insecticide treated animals to untreated animals. Reduction in weight gains up to 67% have been demonstrated (Laake, 1946; Bruce and Decker, 1951; Cheng, 1958; Cutkomp and Harvey, 1958; Koehler and Butler, 1976). In Nebraska, calves from cows without horn fly populations gained 5.4-6.3 kg over calves from untreated cows (Campbell, 1976). Recent work by Butler and Koehler (1977 and 1979) in Florida demonstrated statistically significant differences in weight gain from 10-72% as a result of residual sprays and dust bag control treatments; treated animals gained 0.06-0.35 kg per day over untreated animals with high fly populations.

Fly populations as low as 50 per side have been reported as being economically damaging (Lofgren, 1970). In Florida, untreated animals may carry an average of 800 flies per animal for up to 9 months of the year (Butler and Koehler, 1979). Estimated losses in the United States of over $197 million to the livestock industry have been estimated to occur per year (Knipling, 1972; Graham and Hourrigan, 1977); other estimates place the losses in control costs and production losses to be in excess of $365 million annually (Anonymous, 1976). In Florida, Butler (1975) estimated an annual primary market loss of $35,382,758 if no horn fly control was conducted in the state.

Disease transmission. Experimental evidence for disease transmission by horn flies is minimal (McClintock and Depner, 1954; Greenberg, 1971 and 1973). But horn flies have been shown to transmit anthrax in the laboratory. Morris (1918) obtained infections of anthrax in sheep and guinea pigs fed upon for 1-3 minutes by horn flies which had previously fed upon guinea pigs infected with Bacillus anthracis; however, horn flies were not shown to feed upon anthrax carcases naturally. Glaser (1924) provided data indicating that Trypanosoma theileri could be transmitted by H. irritans. Trypanosoma congolense and the poliovirus have been reported uncommonly in association with horn flies (Greenberg, 1971). The horn fly is additionally an intermediate host of Stephanofilaria stelesi, a filarial nematode that causes skin lesions on the underside of cattle (Hibler, 1966). McClintock and Depner (1954) cited references indicating that the closely related genus, Lyperosia, contains species suspected of transmitting trypanosomes; nevertheless, horn flies themselves have not yet been implicated.

Hypothetically, the horn fly has a number of characteristics providing a high potential for disease transmission: they feed frequently on cattle; feeding is interrupted in nature because of host response to bites (Zumpt, 1973); and horn flies have been shown to move extensively within a given herd (see section, "Flight Behavior"). Butler et al. (1977) indicated that horn flies may be potential mechanical vectors of diseases transmitted by contaminated mouthparts or infected feces.

Control. Early efforts to control horn flies by scattering manure or using various repellants were generally ineffective. Bruce (1938) developed a moderately effective trap for horn flies which was situated so that infested animals were forced to walk through the trap. Modern residual insecticides applied in self-applicating devices such as back and face rubbers, back oilers, and dust bags have proven to be effective. Insecticide treated collars, blocks, and ear tags are also effective (Rogoff and Moxon, 1952; Lindquist and Hoffman, 1954). Harvey and Brethour (1979) recently demonstrated that treatment of one animal per herd with pyrethrin gave effective control of horn flies. Of the many insect species known to prey upon or parasitize the horn fly, the hymenopterous pupal parasites are the most numerous (Depner, 1968; Greer, 1975; Escher, 1977); however, in natural situations they do not appear to keep horn fly populations below economic levels.

Because current control of horn flies is completely dependent on insecticides with no prospective alternatives available, Knipling (1972) proposed that the feasibility of horn fly eradication be considered. He proposed two strategically timed insecticide applications to reduce fly populations by 99% followed by sterile fly releases for

6 generations totaling 300 released horn flies per animal. He projected a cost of $100 million dollars to achieve elimination of horn flies from the United States, Canada, and Mexico. Sterile male releases in west and central Texas (Kunz et al., 1974) were important in showing that an isolated location was necessary to demonstrate eradication. A 3 year integrated control program on the island of Molokai in Hawaii using a combination of orally administered methoprene and sterile male release apparently demonstrated that horn flies could be eliminated from semi-isolated areas (Eschle et al., 1977). In addition, this pilot study: 1) demonstrated difficulties in mass-rearing of horn flies; 2) suggested problems in combining certain control procedures; and 3) indicated that additional in-depth research was necessary before any large control program could be undertaken (Graham and Hourrigan, 1977).

Courtship and Mating Behavior of the Diptera

General Considerations

Richards (1927) provided one of the earliest reviews of insect reproductive behavior from the perspective of sexual selection. He described how the events in the sexual behavior of a species were thought to be facilitated by a combination of gustatory, aural, visual, tactile, or olfactory stimuli from either sex. According to Guhl and Schein (1968) sexual behavior includes courtship and mating activities. Courtship is "the premating behavior that stimulates one or both individuals and initiates mating performance" (p. 191); mating is defined synonymously with copulation. Guhl and Schein (1968) emphasized that the point at which courtship ends and mating begins can be difficult to determine.

Manning (1966) noted that the occurrence of courtship in insects was varied and sporadic. For example, in the Muscidae and Calliphoridae, males appeared to display little if any courtship behavior in comparison to the elaborate courtship displays of males of certain Drosophilidae species. In many Muscid and Calliphorid species, males will mount dark objects of an appropriate size. Even generalized visual stimuli are not required since mating will occur in the dark. Also, copulation with or rejection by a conspecific female can occur within several seconds. Although this brief period between mounting and attempted copulation did not seem to allow much time for the exchange of complex communication between the sexes, recent investigations have demonstrated that Muscid males do perform a series of complex courtship actions (Ewing, 1977). Bastock (1967) stated that arthropod courtship was surprisingly complex, but was not evoked in any animal with mechanical consistency.

Chapman (1969) and Matthews and Matthews (1978) have reviewed general considerations of insect reproductive behavior. Parker (1978a) listed many of the conventional encounter sites where males and females meet before initiating courtship behavior. Anderson (1974) reported that the mating activity of many arthropods of medical-veterinary importance including many Diptera are closely associated with a host. Of these arthropods, least is known about the mating behavior of the Cyclorrhapha.

Although authors such as Richards (1927), Alexander (1964), Bastock (1967), and Matthews and Matthews (1978) have attempted to consider insect courtship behavior in an evolutionary perspective, there has been a more recent attempt to evaluate insect reproductive behavior in

terms of modern sexual selection theory by a number of investigators (Halliday, 1978; Parker, 1978b; Barrass, 1979; Blum and Blum, 1979; Borgia, 1979; Otte, 1979). A noteworthy example of research conducted with this theory in mind is a series of papers on the dung fly, Scatophaga stercoraria, by Parker (1969, 1970a, 1970b, 1970c, 1970d, 1970e, 1971a, 1971b, 1974). Not only has this research provided experimental data to develop models for sexual selection theory, but it also represents an excellent example of a field study on the reproductive behavior of a Dipteran species.

Courtship and Mating Behavior of the Muscidae

The horn fly. Few details concerning the courtship or mating behavior of the horn fly, Haematobia irritans (L.), have been reported. Bruce (1940, 1942) stated that mating flies had been observed in the field as early as the second day after emergence. Hammer (1941) had reported seeing copulating pairs of horn flies on the sides of cows; however, he did not describe the courtship sequence preceding copulation. Bruce (1964) noted that mating occurs on host animals and that copulating pairs had been seen on vegetation near the host. Mating pairs remained in copulation about 0.5 to 5.0 minutes. Anderson (1974) listed Haematobia irritans as a species that mated on the host based on the unpublished data from California of J.R. Anderson, E.C. Loomis, and R. Fontaine. Courtship behavior was observed on cows but not in the air around the host; males would jump or walk onto resting females and if successful in copulating would remain in copulo with the female on the host (Anderson, pers. comm., 1979, Division of Entomology and Parasitology, University of California, Berkeley, CA). The data of

Harris et al. (1963) suggested that the horn fly is monogamous but males inseminated an average of 4.6 females and up to as many as 8 females. In the laboratory, mating occurred as early as 2 days after emergence; most flies had mated by the end of the fourth day; however, when flies were placed on a cow, mating began as early as 1 day after emergence, and all flies had mated by the end of the second day.

The face fly. The mating habits and courtship behavior of the face fly, Musca autumnalis DeGeer, have been investigated by a number of researchers. Hammer (1941) noted that conspicuous objects in fields such as a rock, cow, or cart were probable mating sites. Resting males would catch females flying by. After ascending slightly as a couple, they would fall to the ground to complete mating. Field observations by Teskey (1969) confirmed Hammer's descriptions. Ode and Matthysse (1967) observed mating pairs of face flies on the sunny sides of farm houses in April after flies had emerged from hibernation. Female face flies have been reported to mate once by Teskey (1960, 1969) and Jones (1964) but more than once by Wang (1964). Killough and McClellan (1969) observed that most females mate only once, but that those females attempting to mate again had not obtained sperm during the first copulation. Males mate repeatedly (Teskey, 1969) with an average of 4 females (Lodha et al., 1970).

Most matings occurred between flies which were 3-7 days old (Wang, 1964; Teskey, 1969; Lodha et al., 1970) but mating can begin 36 or 48 hours after pupal eclosion for males and females, respectively (Lodha et al., 1970). Chaudhury and Ball (1974) noted that the period of maximum attraction and insemination between mature virgin females (4-8 days old) and mature unmated males (5-6 days old) occurred around noon

and reached a minimum in the evening. Various authors have reported the following times for the duration of copuation: 5 minutes-4 hours (Wang, 1964); 60-85 minutes (Teskey, 1969); 44-76 minutes (Killough and McClellan, 1969); and 25-135 minutes (Lodha et al., 1970). The average copulation duration necessary for effective sperm transfer was 66.2 minutes; sperm transfer began after the first 4 minutes of copulation (Lodha et al., 1970).

Teskey(1969) and Lodha et al. (1970) described some aspects of the courtship behavior of face flies; however, Tobin and Stoffolano (1973b) presented a detailed analysis of face fly courtship behavior and compared the courtship behavior of both the face fly and house fly. In a separate study, Tobin and Stoffolano (1973c) investigated how differences in the courtship behavior of face flies and house flies may play a role in preventing hybridization between these closely related species.

Fannia species. The mating behavior of several species of the genus Fannia has been described. Tauber (1968) reported details of the courtship sequence of both Fannia femoralis Stein and F. canicularis (L.). In several studies, Koyama (1962a, 1962b, 1974) has invesitaged the sexual and physiological aspects of mating behavior of F. scalaris Fabricius.

Male F. femoralis walk, jump, or fly onto the female's dorsum beginning a sequence of precopulatory behavior similar to those of F. canicularis; however, initial contact between the sexes of F. canicularis can occur in flight (Tauber, 1968). Anderson (1974) cited his unpublished data indicating that F. canicularis and F. femoralis mate at the emergence sites on poultry ranches in California.

F. femoralis females mate only once unless the previous mating was infertile. Both the length of courtship and the termination of copulation appear to be controlled by the female. A non-receptive virgin or previously mated female thwarted the advances of males by (1) kicking with her legs; (2) arching her abdomen downward; (3) holding her wings overlapped on her back; or (4) raising and rapidly moving her wings (Tabuer, 1968).

F. scalaris Fabricius were observed swarming and mating under willow trees by Koyama (1962a); only physiologically mature males displayed mating behavior. Although these males indiscriminately pursued males and females of different ages, only mature males and middle-aged virgin females copulated. Trial and error searching by males for females during precopulatory swarming appeared to be the primary way that males located females (Koyama, 1962a, 1962b, 1974).

The house fly. The courtship behavior of the house fly, Musca domestica L., has been extensively studied by numerous investigators; West (1951), Tobin (1972), and Colwell (1973) review many of the early descriptive reports on house fly reproductive behavior. As well as reviewing more current studies on house fly courtship, a number of recent papers have presented detailed descriptions of house fly courtship (Tobin and Stoffolano, 1973a; Colwell and Shorey, 1975, 1976, and 1977) which were obtained using a combination visual observation as well as still and motion picture photography.

A photographic analysis of house fly courtship by Tobin and Stoffolano (1973a) provided a detailed description of the courtship elements and time ranges for each element between virgin males and virgin females. These females had been anesthetized prior to testing

and tethered in place with an insect pin placed through the thorax. Barriers to hybridization caused by differences in courtship sequences between house flies and stable flies were also investigated by Tobin and Stoffolano (1973c).

Colwell and Shorey (1975) described the stereotyped courtship of untethered, virgin house flies as follows: While vibrating his wings, the male jumps or flies onto the dorsum of the female whose wings are extended to a horizontal position and whose metathoracic legs are raised behind her wings. As the male moves anteriorly on the female, he may twist his head to one side and/or extend his proboscis. After reaching down with his prothoracic legs and manipulating the female's prothoracic legs, he moves backward on the female and attempts to copulate. Colwell and Shorey (1975) suggested that inconsistencies in existing descriptions of house fly courtship may be due to genetic, physiological, or environmental differences among strains. Their study clarified many conflicting descriptions of the elements of courtship by also emphasizing that variability exists in behavior displayed by each sex during courtship and by suggesting how experimental conditions can alter behavioral responses.

Experiments by Colwell and Shorey (1976), in which house flies were reared without light, confirmed earlier reports that elimination of visual stimuli impeded the rate of mating but did not prevent mating. In addition, they reported that wingless males could still copulate with females but that the courtship duration was lengthened. The time to initiate copulation was greatly increased when both males and females had various appendages amputated which hypothetically interfered with the male's production or the female's reception of stimuli important

in the continuation of courtship. Murvosh et al. (1964) previously had reported similar although less detailed findings.

In another investigation, Colwell and Shorey (1977) determined that males could distinguish between tethered males and tethered females even though a variety of visual, acoustical, and behavioral differences between the sexes were eliminated. Female-produced chemical(s) perceived by both olfaction and contact chemoreception appeared to be important in stimulating males to continue through the entire courtship sequence. The number of courtship responses between live males and dead males increased with the age of the dead male while the number of courtship responses between live males and dead females decreased with the age of the dead female. At 20 days of age, no differences existed between the number of courtship responses elicited by dead males and females; therefore, Colwell and Shorey (1977) hypothesized that early termination of courtship on normal males may be due, in part, to inhibitory chemicals associated with males while continuation of courtship on normal females may be due to excitatory chemicals which are gradually lost after death.

Chabora (1978) reported reduced rates of courtship and mating in a green-eyed, visually deficient mutant strain. Reciprocal crosses between this deficient strain and a normal strain indicated that a visual component appeared to be involved in the courtship of both males and females. In addition, no evidence for a rare male advantage was found which agreed with the previous findings of Childress and McDonald (1973).

Zingrone et al. (1959) had established that most females are monogamous with only 2% of the females mating a second time. Cowan

and Rogoff (1968) demonstrated that individual males varied in their mating responsiveness to control pseudoflies and pseudoflies treated with benzene extracts of female flies. A greater number of flies responded to treated pseudoflies than to controls. The activity of individual responding flies to treated pseudoflies also occurred. The response tendencies of males appeared to be inherited.

The stable fly. The mating behavior of the stable fly, Stomoxys calcitrans (L.),has been investigated in a number of separate studies (Parr, 1962; Killough and McKinstry, 1965; Harris et al., 1966; Muhammed, 1975; Anderson, 1978). Field studies in Uganda by Parr (1962) indicated that stable flies mated when each sex was 6 days old; yet Killough and McKinstry (1965) reported that 1-day old males or females in the laboratory could mate with 5-day old individuals of the opposite sex. The data of Harris et al. (1966) showed that a few male and female stable flies mated as early as 2 days after emergence; most had mated by the fifth day. The female stable fly was reported to mate only once, but the male mated as many as 9 females.

Muhammed (1975) compared and contrasted the sexual behavior of laboratory stable flies with the descriptions made by Tobin and Stoffolano (1973a) for the house fly. Of the seven generalized stages of interactions which were observed between virgin males and virgin females, he identified that head touching of the female by the male's proboscis while the male positioned himself on female was the most important element in stable fly courtship; this element occurred more frequently than others and was reverted to and repeated if later events in the courtship sequence were disturbed. The "boxing" stage in house fly courtship described by Tobin and Stoffolano (1973a) in which the male's and

female's prothoracic legs touch was not observed in the stable fly.

Anderson (1966) described basic aspects of stable fly courtship which were more fully elaborated upon in a recent report (Anderson, 1978). He described that the general mating behavior of properly nourished, sexually mature stable flies observed in laboratory cages was similar to the courtship behavior of the house fly and face fly as described by other researchers. His descriptive account of the sequence of courtship events between aggressive, virgin males and receptive and nonreceptive virgin females included details of how the male positions himself on the female's dorsum and how the legs and wings of each sex are positioned. No mention was made in this account of touching the female's head by the male's proboscis as described by Muhammed (1975).

By observing male and female pre-mating behavior in the field, Bushman (pers. comm., 1978, Insects Affecting Man and Animals Research Laboratory, USDA, Gainesville, FL) suggested that the initial male-female contact in nature usually occurs in flight. This in-flight contact precedes the courtship sequences described by most researchers who observe mating behavior of caged laboratory flies. Muhammed (1975) had noted that on occasion males did intercept females in flight in laboratory observation cages. After the male and female fell to the cage bottom, courtship behavior was resumed. Hammer (1941) had also described field observations of this phenomenon for a number of genera including Stomoxys.

The tsetse flies. Mellanby (1936) observed that mating occurred rapidly in the laboratory when virgin male and female Glossina palpalis(R.D.)

were placed together. Often many males would court 1 female until 1 male succeeded in copulating with her. Males would then court the next most attractive female while some females were left uncourted. Squire (1951) described the general events occurring during the courtship of G. palpalis (R.-D.). A male would quickly mount a female who had opened her wings. The male's hypopigium gripped the female's abdomen and the claspers grasped the copulatory cushions on the female's sixth segment. Disturbed pairs readily took flight with the female essentially carrying the male along with her.

G. palpalis fuscipes males can seize females which are resting, feeding, or flying. When copulating, the male is on the female's dorsum; her wings are slightly spread and bent. The tarsi of the prothoracic legs grasp the female between the head and thorax, the male's mesothoracic legs vary in position, but the male's metathoracic legs trail behind or rest upon the female's wings. Before separating, copulating pairs begin to move around (Buxton, 1955). Dean et al. (1969) noted that virgin female G. morsitans orientalis Vanderplank with partly opened wings passively accept males. Squire (1952) noted that G. palpalis females were often seized by males in mid-air and grasped with the claspers.

In G. morsitans orientalis, laboratory reared males are visually attracted to moving objects and apparently can not distinguish females until within 1-2 cm; females do not appear to seek out males. Squire (1951) described the position of the male and female genitalia during copulation of G. palpalis, and Pollock (1973) related the internal anatomy of G. austeni Newstead females to copulatory process.

A number of investigators have described the means by which females reject advances by males. Mellanby (1936) stated that it appeared impossible for males to copulate with a reluctant female G. palpalis which had closed its wings and bent its abdomen downward. Squire (1951) and Jordan (1958) observed that G. palpalis females repelled male courtship attempts with vigorous wing movement or closed wings. Mated females of Glossina morsitans orientalis also thwarted further copulatory attempts by males by bringing the wings together, by curving the abdomen downward, or by shaking the male off of her dorsum.

Nash (1955) reported that some G. palpalis pairs will mate within 24 hours of emergence. In G. morsitans orientalis the female's willingness to accept males decreases as the number of successful or unsuccessful copulatory attempts increases (Dean et al., 1969); females copulating less than 45 minutes received no sperm. Rogers (1973c) reported that the copulation duration of G. pallidipes Austen averaged less than 26 minutes and decreased in duration slightly with older females; other Glossina sp. rarely copulate less than 60 minutes. The copulation duration of G. palpalis decreases with the age of the female as follows: 1-3 day old, 2 hours; 4-9 days old, 75 minutes; and 10 days old, less than 1 hour (Jordan, 1958).

Squire (1951) claimed that G. palpalis (R.-D.) females may be mated many times; however, a single fertilization can provide the female with sufficient sperm for her lifetime (Buxton, 1955). Jordan (1958) demonstrated that remating was most frequent with young G. palpalis females. Dame and Ford (1968) cited evidence for multiple mating of both males and females of G. morsitans in the laboratory but noted that the frequency of remating in nature was unknown. Rogers

(1973a) reported that of 109 female G. pallidipes caught on a bait animal, 13 were previously inseminated but 2 females were only partly inseminated. In the laboratory, 75 G. pallidipes males were repeatedly mated; 32 males mated 1 time; 15 males mated 2 times; 3 males mated 3 times; and 1 male mated 4 times in 5 hours (Rogers, 1973b).

Foster (1976) reviewed the conflicting reports concerning the age at which males of different Glossina species become sexually mature and presented data demonstrating that blood feeding affects sexual maturation differently in G. morsitans than in G. austeni. Tobe and Langley (1978) reviewed general considerations of mating, receptivity, and reproductive physiology of Glossina while Mulligan (1970) reviewed general aspects of copulation and insemination of the genus.

Vanderplank (1948) reported on the sound produced during mating of Glossina sp. Dean et al. (1969) demonstrated that elimination of sound in mating pairs of G. morsitans orientalis by glueing the wings and halteres did not alter mating success, but increased the time from pairing of the sexes until copulation. Ultrasound components (30-70 kHz) produced by mating pairs of G. morsitans differ from feeding sounds produced by each sex (Erickson and Moller, 1975). Rudrauf (1977) reported that courting pairs of G. fuscipes fuscipes Newstead emitted high-pitched frequency signals up to 80 kHz before and between mating attempts; these signals were not detected from non-courting pairs.

Sex Pheromones of Diptera

General Considerations

In 1971, Law and Regnier proposed the term semiochemical (GK. simeon, a mark or signal) to describe those chemicals involved in the chemical interaction between organisms. The term pheromone (GK. pherein, to carry, and horman, to excite or to stimulate) was coined by Karlson and Butenandt in 1959 to describe chemicals involved in intraspecific interactions (Nordlund and Lewis, 1976). Within the last 5 years rapid progress has been made in practical research directed toward the development of pheromones and other behavior-modifying chemicals for insect pest management (Brand et al., 1979).

The use of pheromones by insects has been the most extensively studied form of chemical communication (Young and Silverstein, 1975) as is evidenced by the reviews on insect pheromones that have recently been published in the form of books (Wood et al., 1970; Beroza, 1970; Jacobson, 1972; Birch, 1974; Jacobson, 1975; Beroza, 1976; Inscoe and Beroza, 1976; Shorey, 1976; Shorey and McKelvey, 1977) or review articles (Shorey, 1973; Mayer and McLaughlin, 1975; Shorey, 1977; Roelofs, 1978; Weaver, 1978; Brand, 1979). In particular, Young and Silverstein (1975) provide an excellent review of important considerations in the biological and chemical methodology in the study of insect communication. Seabrook (1978) reviewed neurobiological contributions related to understanding insect pheromone systems. Because insect pheromones can be common components of the cuticular lipids, the review of insect waxes by Jackson (1976) is informative.

In general, insects can be highly selective and sensitive to sex pheromones (Young and Silverstein, 1975). But unlike the sex pheromones of Lepidoptera, those of the Diptera are usually not a sufficient stimulus by themselves to evoke male copulatory behavior without simultaneous visual stimuli (Shorey, 1973). The ability of Dipterous female sex pheromones to attract males from a distance is low. Males tend to aggregate in the vicinity of females due to other stimuli such as environmental conditions (Shorey, 1973). Anderson (1974) pointed out that for all arthropods of medical and veterinary importance including the Diptera, pheromones do not play a major role in bringing the sexes together, but rather function in recognition of the sexes once they have met at a given site.

In his review of the behavioral responses of Diptera to pheromones and other semiochemicals, Fletcher (1977) reported that most pheromones appear to be complex blends of compounds. Tamaki (1977) addressed the problem of complexity, diversity, and specificity of pheromones and semiochemicals in Diptera and Lepidoptera. When considering species associated with a host, Young and Silverstein (1975) suggested that synergism of a pheromone may involve compounds emanating from the host.

Several species of Dipteran pests of fruit and vegetable crops have been controlled with baits consisting of an attractant and pesticide or with attractant baited traps (Brand, 1979; Roelofs, 1979). Of the Dipteran pests that directly affect man or animals, pheromones have been identified and field-tested in only a few cases. The house fly pheromone, muscalure, is one of only two pheromones presently registered by EPA for use in pest control (Brand, 1979; Roelofs, 1979).

Sex Pheromones of the Muscidae

The house fly. The report by Carlson et al. (1971) that (Z)-9-tricosene, commonly called muscalure, was a sex attractant pheromone of the house fly, Musca domestica L., represented the first isolation, identification, and synthesis of a sex pheromone from the family Muscidae. Since that report, sex pheromones, primarily mating stimulant compounds, of other species in the family have been reported as summarized in Table 2-1.

A number of independent studies had preceded the identification of muscalure. Rogoff et al. (1964) demonstrated the presence of a house fly sex pheromone by the attraction of males to females in olfactometer trials and by the stimulation of males to mate with pseudoflies treated with extracts of female flies. These studies were confirmed by Murvosh et al. (1965) while Mayer and Thaggard (1966) provided additional data in support of the presence of a sex attractant. Mayer and James (1971), Silhacek et al. (1972a and 1972b), and Voaden et al. (1972) accomplished partial isolation of the active compound from the non-polar lipid fraction. After the identification of muscalure as the sex attractant by Carlson et al. (1971), Rogoff et al. (1973) confirmed that muscalure also functioned as a mating stimulant for male house flies.

Several later investigations indicated that (Z)-9-tricosene was not highly specific and was not the only natural compound responsible for releasing the sexual behavior of male house flies. A number of structural analogs of muscalure tested by Carlson et al. (1974) were as active as muscalure. In 1972, Mansingh et al. reported active members of a series of C_{19} to C_{25}(Z)-9-alkenes which induced and maintained

high excitement and mating behavior of male house flies. The greatest activity demonstrated was for a ratio of (Z)-9-tricosene (70%) and (Z)-9-heneicosene (30%) which were hypothesized to be a mating stimulant and attractant, respectively; however, these findings could not be confirmed by Carlson et al. (1974). The enhancement of mating strike activity by the combination of certain methyl-branched C_{27} and C_{29} paraffins of female origin was documented by Uebel et al. (1976). Carlson et al. (1974) suggested that the low specificity of Dipteran sex pheromones such as muscalure versus sex pheromones of Lepidoptera may be characteristic of sex pheromones that are not highly potent.

In field evaluations, Carlson and Beroza (1973) found that muscalure increased house fly catches in several types of fly traps. Unexpectedly, both females and males were attracted to muscalure baited traps indicating that muscalure might act as an aggregation pheromone for both sexes. Morgan et al. (1974), while testing (Z)-9-tricosene in the field in sugar bait formulations, also found that muscalure was an attractant for both sexes. A field study in open poultry houses by Mitchell et al. (1975) demonstrated that the addition of (Z)-9-tricosene to baits in fly traps increased the capture of house flies 2 to 14 times over traps without (Z)-9-tricosene in the bait. The sex ratios of flies caught in both treated and untreated traps was similar.

Uebel et al. (1978d) reported that the total non-hydrocarbon fraction of female house flies may be active at 100 and 200 µg in stimulating males to copulate with treated pseudoflies. Isolation and identification of three major non-hydrocarbon fractions led to the discovery that two of these compounds were as active or more active at 100 and 200 µg in pseudofly tests than was (Z)-9-tricosene. The

authors hypothesized that the unsaturated ketone, (Z)-14-tricosen-10-one, and the epoxide, (cis)-9,10-epoxytricosane, were involved in the sex pheromone complex of the house fly.

The face fly. Attraction and mating stimulation of male flies by a volatile sex pheromone extracted from mature female face flies (Musca autumnalis De Geer) was reported by Chaudhury et al. (1972). The pheromone was tentatively thought to be an unsaturated hydrocarbon. Uebel et al. (1975a) isolated and identified the active components of the pheromone including the straight-chain monoalkenes (Z)-14-nonacosene, (Z)-13-nonacosene, and (Z)-13-heptacosene. Pheromone activity was bioassayed by counting mating strikes made by virgin males upon other virgin males treated with 100 µg, 200 µg, or 300 µg of selected mono-olefins. Treated males were impaled on pins taped to soda straws which were introduced into quart Mason jars holding a set number of virgin males. The number of strikes made upon these treated males versus the strikes upon an impaled female served as the basis for evaluating pheromone activity. Both male and female adults were shown to have about equal quantities of these active cuticular compounds; however, males had higher concentrations of saturated hydrocarbons, especially heptacosane and nonacosane, which reduced the activity of the previously listed alkenes. Because females had a lower proportion of saturated hydrocarbons and a higher proportion of active unsaturates than did males, the authors proposed that males may have a means to differentiate the sexes.

Using the bioassay described above, Sonnet et al. (1975) evaluated combinations of synthetic compounds, originally determined in male and female face fly cuticular washes, along with other selected synthetic

compounds in an attempt to optimize the activity of the most active natural compound (Z)-14-nonacosene. No combination was found to be more active than (Z)-14-nonacosene although several inhibitory compounds were identified. Sonnet et al. (1975) mistakenly reported that Uebel et al. (1975a) had found nonacosene and heptacosene to be inhibitory; in fact, Uebel et al. (1975a) had reported that nonacosane and heptacosane were inhibitory.

The stable fly. Using a combination of bioassay techniques to investigate possible pheromones of the stable fly, Stomoxys calcitrans (L.), Muhammed et al. (1975) reported that trans and cis olefins were the mating stimulant compounds for males. Female polyolefins were reported as a sex attractant for males. Uebel et al. (1975b) found that a number of saturated and unsaturated female hydrocarbons were the mating stimulant for the male. The active saturates included mono- and dimethyl-substituted hentriacotanes and tritriacontanes while (Z)-9-hentriacontene, (Z)-9-tritriacontene, and methyl-branched hentriacontenes and tritriacontenes were the main active unsaturates. Sonnet et al. (1977) continued work on the saturated hydrocarbons by synthesizing and testing several dozen methyl-branched and 1,5-dimethyl-branched alkanes. Bioassay results indicated that the 15-methyl and 15,19-dimethyltritriacontanes had the greatest mating stimulant activity.

Both Uebel et al. (1975b) and Sonnet et al. (1977) used the Mason jar bioassay which measured strikes upon treated, impaled males as was previously described for the face fly studies conducted by Uebel et al. (1975a). Treatment dosages of 50, 100, 150, or 200 µg were tested. Sonnet also used a bioassay designed by Harris et al. (1976) in which

strikes of male stable flies upon treated male house flies were mechanically recorded. Bioassays by Sonnet et al. (1979) utilized hexane-rinsed male house flies as the pseudofly rather than male face flies to avoid interference in bioassays due to the natural polyolefin present on male face flies.

Additional work by Sonnet et al. (1979) indicated that the stable fly sex pheromone which induces male copulatory behavior was a complex of compounds which were most active in combination. Principal components included (Z)-9-hentria- and tritriacontenes; 13-methyl-1-hentri- and tritriacontenes as well as an array of methyl-branched hentria and tritriacontanes, with 11- and 15-methyl-substituted compounds being the most active.

Fannia species. Uebel et al. (1975c) identified (Z)-9-pentacosene as the hydrocarbon which stimulated the male little house fly, Fannia canicularis (L.), to mate. This compound represented the major portion of the cuticular hydrocarbons of the female and appeared to be more specific in eliciting male copulatory response than did the sex pheromones of other Muscidae (Uebel et al., 1977). Unbranched monoolefins of 31 and 33 carbon length were described by Uebel et al. (1978a) as active mating stimulant compounds from female F. pusio (Wiedemann). A major C_{31} monoolefin, (Z)-11-hentriacontene, found in the female but not in the male, was found to be the most active of synthetic compounds bioassayed. The same compound was the most active male mating stimulant of F. femoralis (Stein) tested by Uebel (1978b). The addition of female alkanes increased the activity of tested C_{31} monoolefins. The primary difference in the sex pheromone makeup of F. pusio versus F. femoralis appears to be the synergistic action of female alkanes

occurring in F. femoralis but apparently not in F. pusio. Males of either species will, however, attempt to copulate with females of the other species if the two sexes are placed together in a confined environment.

The bioassays utilized in these studies on species of Fannia were all conducted as described by Uebel et al. (1975a) with treatment dosages of 100 and 200 µg. Only mating strikes were recorded in these bioassays. Males displaying other courtship behavior were physically removed from the treated pseudofly.

In simulated field trials, the respective mating stimulant pheromone of F. canicularis, (Z)-9-pentacosene, and F. pusio, (Z)-11-hentriacontene, were moderately effective in attracting males to pheromone treated baits. The mating stimulant of F. femoralis, (Z)-11-hentriacontene, was ineffective in attracting either sex of that species (Uebel et al., 1978c).

The tsetse fly. Langley et al. (1975) reported evidence for the existence of a sex-recognition pheromone in the tsetse fly, Glossina morsitans morsitans (Westwood). Located in the non-polar cuticular lipid fraction of the female, the pheromone appeared to be active only at short distances. The active component was hypothesized to be a long chain hydrocarbon with a carbon number of C_{31} to C_{38}. Carlson et al. (1978) later isolated, identified, and synthesized three sex recognition components which are naturally produced by the females and which stimulate males to initiate sexual behavior upon contact with the female. The compounds 15,19-dimethylheptatriacontane, 17,21-dimethylheptatriacontane, and 15,19,23-trimethylheptatriacontane were reported to be the least volatile and most stable pheromones reported to date. Field

testing of these compounds in Tanzania confirmed the stimulatory activity of the most active isomer on wild males and established increased efficiency of currently-used survey methods when supplemented with pheromone (Dr. D. Carlson, pers. comm., 1978, Insects Affecting Man and Animals Research Laboratory, USDA, Gainesville, FL).

The horn fly. Mackley (1977) extracted, purified, and examined horn fly lipids for possible pheromone activity as attractants or contact stimulants. Olfactometer studies demonstrated a low order of attraction of about 1.7x of sexually mature female horn flies to the total monoolefin fraction than to blank controls. Contact stimulation assays indicated that horn fly courtship behavior was, in part, chemically mediated; however, no active compounds were identified because of the lack of a suitable bioassay.

Table 2-1. A summary of sex pheromone research on the Muscidae.

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Musca domestica L. (house fly)				
	(Z)-9-tricosene	olfactometer	sex attractant for males	Carlson, Mayer, Silhacek, James, Beroza, and Bierl, 1971
	7:3 ratio of (Z)-9-tricosene and (Z)-9-heneicosene	olfactometer	mating stimulant and sex attractant for males	Mansingh, Steele, Smallman, Meresz, and Mozogai, 1972
	(Z)-9-tricosene	pseudofly	mating stimulant for males	Rogoff, Gretz, Jacobson, and Beroza, 1973
	(Z)-9-tricosene	field trial-baited traps	attractant for males and females	Carlson and Beroza, 1973
	(Z)-9-tricosene and analogs	olfactometer, pseudofly	sex stimulant as well as sex attractant for males	Carlson, Doolittle, Beroza, Rogoff, and Gretz, 1974
	(Z)-9-tricosene	field trial	attractant for males and females	Morgan, Gilbert, and Fye, 1974
	(Z)-9-tricosene	field trial	attractant for males and females	Mitchell, Tingle, and Carlson, 1975

Table 2-1. Continued

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Musca domestica L. (continued)				
	(Z)-9-tricosene plus methyl- and dimethyl-branched C_{27} to C_{29} paraffins of female origin	pseudofly	mating stimulant for males	Uebel, Sonnet, and Miller, 1976
	(Z)-14-tricosen-10-one and cis-9,10,epoxy-tricosane	pseudofly	stimulated copulatory responses from males	Uebel, Schwarz, Lusby, Miller, and Sonnet, 1978d
Musca autumnalis De Geer (face fly)				
	female face fly, unsaturated hydrocarbons	olfactometer, female face fly models	sex attractant and sex stimulant for males	Chaudhury, Ball, and Jones, 1972
	(Z)-14-nonacosene (Z)-13-nonacosene (Z)-13-heptacosene	treated male face flies	mating stimulant for males	Uebel, Sonnet, Miller, and Beroza, 1975a
	(Z)-14-nonacosene	treated male face flies	mating stimulant for males	Sonnet, Uebel, and Miller, 1975

Table 2-1. Continued

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Stomoxys calcitrans (L.)				
	female polyolefin fraction	olfactometer, pseudofly, activity meter	sex attractant for males	Muhammed, Butler, and Carlson, 1975
	(E) and (Z) olefins	olfactometer, pseudofly, activity meter	mating stimulant for males	. .
	saturated hydrocarbons: mono- and dimethyl-substituted hentria- and tritriacontanes	treated male stable flies	mating stimulant for males	Uebel, Sonnet, Bierl, and Miller, 1975b
	unsaturated hydrocarbons: (Z)-9-hentriacontene (C_{31}); (Z)-9-tritriacontene (C_{33}); methyl-branched hentria- and tritriacontenes	treated male stable flies	mating stimulant for males	. .

Table 2-1. Continued

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Stomoxys calcitrans (L.) (continued)	11,15-dimethylhentriacontane; 15-methyltritriacontane; 15,19-dimethyltritriacontane	treated male stable flies, male house flies with recording device	mating stimulant for males	Sonnet, Uebel, Harris, and Miller, 1977
	(Z)-9-hentriacontene; (Z)-9-tritriacontene; 13-methyl-1-hentriacontene; 13-methyl-1-tritriacontene	male house flies rinsed with hexane	mating stimulant for males	Sonnet, Uebel, Lusby, Schwarz, and Miller, 1979
Fannia canicularis (L.) (little house fly)	(Z)-9-pentacosene	pseudofly	mating stimulant for males	Uebel, Menzer, Sonnet, and Miller, 1975c
	(Z)-9-pentacosene	pseudofly	mating stimulant for males	Uebel, Sonnet, Menzer, Miller, and Lusby, 1977

Table 2-1. Continued

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Fannia pusio (Wiedemann)	unbranched C_{31} to C_{33} mono-olefins especially (Z)-11-hentriacontene	pseudofly	mating stimulant for males	Uebel, Schwarz, Menzer, and Miller, 1978a
Fannia femoralis (Stein)	C_{31} to C_{33} mono-olefin especially (Z)-11-hentriacontene	pseudofly	mating stimulant for males	Uebel, Schwarz, Miller, and Menzer, 1978b
Fannia canicularis (L.)	species' respective mating stimulants as listed above	simulated field trial-baited traps	slight attractant for males	Uebel, Schwarz, Sonnet, Miller, and Menzer, 1978c
F. pusio (Wiedemann)			slight attractant for males	
F. femoralis (Stein)			not attractive to males	
Glossina morsitans (Westwood) (tsetse fly)	long chain hydrocarbons	pseudofly	mating stimulant for males	Langley, Pimley, and Carlson, 1975

Table 2-1. Continued

Species	Compound(s)	Experimental Bioassay	Type of Pheromone or Induced Behavior	Citation
Glossina morsitans (Westwood) (tsetse fly) (continued)	15,19-dimethyl-heptatriacontane; 17,21-dimethyl-heptatriacontane; 15,19,23-trimethyl-heptatriacontane	pseudofly	sex stimulation and recognition by males	Carlson, Langley, and Huyton, 1978
Haematobia irritans (L.) (horn fly)	horn fly circular monoolefins	olfactometer	attractant for females	Mackley, 1977

CHAPTER 3

THE COURTSHIP BEHAVIOR OF THE ADULT HORN FLY, *Haematobia irritans* (L.), UNDER LABORATORY CONDITIONS

Abstract

The courtship behavior of mature, well-nourished horn fly males and females was similar to that of other muscids; however, unlike other muscids during one element of the courtship sequence, the male rubs the female's head with its protarsi. The duration of courtship and copulation was variable among pairs. Virgin males courted tethered, virgin females with greater frequency than they courted tethered, virgin males. Virgin males could readily differentiate females from males and court females regardless of whether the horn flies were live, dead, winged, or wingless.

Introduction

Research on the horn fly, *Haematobia irritans*, has provided information on many facets of the biology, behavior, and autecology of this species; however, descriptive literature on horn fly courtship is meager. Hammer (1941) reported observing copulating pairs on host animals in the field. Bruce (1964) made similar field observations and noted that mating pairs remained *in copulo* about 0.5-5.0 minutes. Anderson (1974) listed *H. irritans* (L.) as a species that mated on the host. The data

of Harris et al. (1968) suggested that most horn fly matings occurred between 2-4 days after emergence under laboratory conditions; however, when flies were reared on a host, most flies had mated by the end of the second day. The data of Harris et al. (1968) also suggested that the female horn fly was monogamous.

The basic objectives of this study were to describe the courtship behavior of the horn fly in the laboratory, to obtain a quantitative measure of the behavioral responses occurring between the sexes during courtship, and to investigate selected courtship stimuli which may be important to the male when courting the female.

Materials and Methods

Standard Methods of Rearing and Maintaining Horn Flies

Horn flies used in these studies were from the laboratory colony maintained at the University of Florida (Florida Laboratory Strain, F) as described by Greer (1975). Adults were reared at a temperature of 27 ± 2°C and a relative humidity of 70 ± 10%; continuous lighting for the colony was provided by 6 Westinghouse F40CW (40 W) Cool White fluorescent bulbs. Adults could obtain blood meals at will through the cage top from gauze-covered pads of absorbant surgical cellulose (Doeskin Products, Inc., New York, NY) soaked with bovine blood treated with 0.1 gm of Kanamycin®, 30,000 units of Mycostatin®, and 3.75 gm of sodium citrate per liter. Blood pads on adult cages were changed twice daily.

As adults, flies were considered virgin if they had been sexed within 24 hours of emergence. Flies lightly anesthetized with carbon dioxide were sexed and placed in groups of about 50 flies in 140 ml

clear plastic specimen containers fitted with screen tops. For some experiments individual pupae were placed in 140 ml clear plastic specimen containers. The emerged fly was kept isolated from other flies until testing. In other experiments, male and female flies of a specific age were obtained from dated cages of adults. Because these flies had been held together as mixed sexes for at least 8 days, these flies were considered to be mated when used in experiments. Virgin flies between the ages of 3-9 days were considered sexually mature on the basis of preliminary laboratory observation as well as the data of Harris et al. (1968), Gale (1977), and Mackley (1977).

Methods for Observing Horn Fly Behavior

Visual observation, video tape analysis, still photography, and motion picture photography were used to obtain information on horn fly courtship. Visual observation was initially used to observe general aspects of horn fly behavior in laboratory colony rearing cages and was used in later experiments to count the frequency of specific elements of horn fly courtship. All observations were made at ambient laboratory conditions (25 ± 2°C, RH 70 ± 20%) on the bioassay apparatus described on page 108.

Photographic techniques were used to obtain more detailed information on horn fly courtship. Courting flies were video taped with a Panasonic® TV camera equipped with a S2 110 RND 11.5-110 mm lens (1:2.3) and a +2 accessory close-up lens. Scotch® MBU-40 and MBU-60 video cassettes were analyzed at normal speed and with pause/still frame advance on a JVC video cassette recorder, Model CR-6060 U. Black and white photographs and color slides were taken with a Pentax® ESII 35 mm camera with extension tubes and a Takumar® 100 mm macro lens.

Electronic flash gave an exposure speed of 1/1000 sec. Motion pictures of horn fly courtship sequences were taken with a Fujica® XC-100 super 8 mm camera fitted with a Takumar® 100 mm macro lens and accessory close-up lens on Fuji color movie film (ASA 25 and 200). Film speeds from 18-72 fps and a variable shutter control allowed single frame exposures of 1/40-1/640 sec. Films were viewed at several speeds on an editor-viewer so that individual frames could be analyzed.

The arenas for photography were either a plastic chamber (25 x 20 x 5 mm) fitted with a glass front or a 140 ml clear plastic specimen container fitted with a glass front. Variable back-lighting was used to obtain the proper lighting for the exposures listed above.

Standard Methods of Handling Horn Flies

For photographic purposes or experiments on close-range courtship behavior, horn flies were handled in 2 general ways. In some studies, males and females were provided access to blood until transferred without CO_2 anesthesia to the observation container or photographic arena. Both sexes were free to move about in the container for the duration of the test. In other studies, individual flies were fixed or "tethered" in one location in the observation container or photographic arena. These flies had access to blood until they were "tethered." Under light CO_2 anesthesia, the ventral surface of the abdomen of a fly to be tethered was glued to the head of an insect pin with Weldwood Contact Cement® (U.S. Plywood, Kalamazoo, MI). The pin was inserted into an 8 mm thick, white polyethylene foam sheet (Bernel Foam Products Co., Inc., Buffalo, NY). The excess pin was cut off so that the fly was in a normal standing position with its tarsi in contact with the

foam substrate. Tethered in this manner, flies were able to move all of their appendages. Male horn flies would readily court tethered females. Dead flies for experiments were obtained by freezing the flies at -15°C and thawing them before testing.

Experiments on Courtship Behavior of Untethered Pairs

To observe the courtship behavior of laboratory horn flies, a 4-6 day old virgin male and a 4-6 day old virgin female, each isolated from other flies since emergence, were placed together in a 140 ml clear plastic observation container. The frequencies of the following specific courtship elements were recorded: 1) strike (+), an attempt by the virgin male to get onto the dorsum of the female; 2) arrested movement (++), after striking the female, the responding male remained in contact with the female but did not continue with additional elements of the courtship sequence; 3) positioning on the dorsum (+++), after striking the female, the responding male positioned itself on the female and placed its genitalia below the end of the female's abdomen; and 4) copulation (++++). In addition, certain aspects of these courtship sequences were timed. If copulation did occur, the female was separated from the male and examined to determine if sperm had been transferred. Spermatheca were removed, transferred to a drop of Ringer's saline, and examined for sperm at 100x and 450x both before and after squashing.

Experiments on Female-Produced Stimuli Affecting Courtship of Males

To assess how horn fly males might differently court males versus females, the behavioral responses of groups of 35 virgin males were

observed toward both virgin, tethered males and females. During each of 4 consecutive 15-minute periods 4 elements of courtship behavior were recorded: touch, whenever a male contacted a tethered fly; strike, when a male attempted to get onto the dorsum of a tethered fly; positioning on dorsum, when a male moved backward on the dorsum of the tethered fly and assumed a position for mating; and copulatory attempt, whenever a male placed the ventral portion of the tip of his abdomen against the posterior region of the tethered fly. Tethered flies were randomly assigned to one of 6 predetermined positions on a piece of 8 mm thick, white polyethylene foam circle which fit snugly into the bottom of a 1000 ml glass beaker. The positions of the tethered flies described a circular pattern approximately 7 cm in diameter with each tethered fly facing the center of the beaker.

Under light CO_2 anesthesia, 35 males were transferred to a 140 ml plastic specimen container and given 1 hour to recover from CO_2 before testing began. Without anesthesia, these 35 males were transferred to the beaker containing the tethered flies. The beaker was covered with a clear plastic film containing several dozen air holes. After four 15-minute periods, all flies were discarded. With these same procedures, the courtship behavior between males and females of different ages (3 day old versus 8-9 day old) or females of different mating status (virgin versus mated) was also investigated. Each set of data was analyzed as a split-plot in time analysis of variance. Appropriate F tests or Duncan's multiple range testswere used as tests of significance.

Further experiments to evaluate the importance of female-produced stimuli were conducted by observing the courtship of groups of five 3-6 day old virgin males to both tethered males and females with combinations

of the following treatments: live, dead, winged, and wingless. Flies to be tested were sexed and held in groups until the proper age for testing. Flies for each treatment were tethered as previously described; dead flies were obtained by exposing flies briefly to -15°C temperature. Wings were removed with forceps. The frequency of response to treatments was evaluated by observing if courtship behavior was displayed toward the tethered fly within a 3-minute period after the clear plastic specimen container holding the virgin males was placed over the tethered fly. The first observed courtship behavior was scored as follows: 1) strike (+); (2) arrested movement (++); 3) positioning on dorsum (+++); and 4) copulation (++++). For each treatment, thirty 3-minute trials were performed. The frequency data were analyzed utilizing the chi-square test for contingency tables (Steele and Torrie, 1960; Ostle, 1963).

Results

Preliminary Observation on Horn Fly Behavior in the Laboratory

Visual observation of horn fly behavior in laboratory rearing cages containing several thousand flies or in 120 ml clear plastic cages containing a single pair of virgin flies provided several preliminary findings. Although these observations were qualitative in nature, an overview was obtained of the general activity patterns of horn flies under laboratory conditions (Appendix A-1).

The activity patterns of these flies did change from emergence through several days of age. In particular, the beginning of courtship behavior was not evident until approximately 2.5 days after emergence.

Although few copulating pairs were observed after the fourth day, males continued to court females which became increasingly resistant to male advances and either positioned themselves in locations in the cage which appeared to minimize contact with males or displayed specific rejection behavior. This behavior will be addressed in the next section.

General Description of Courtship Behavior

The courtship behavior of mature, well-nourished H. irritans males and females was similar to that described for other species in the family Muscidae. The following description of horn fly courtship is based on the composite information gained from visual and photographic observations of pairs of courting horn flies in which the female was in some cases tethered and in other cases untethered.

A male horn fly would often orient to and directly approach a female several fly lengths away or at other times would encounter females by seemingly random movement. Orientation to the female was particularly noticeable when the male was on the same surface as the female (Figure 3-1). Males would then walk, jump, or fly into the dorsum of the females. In each of these methods of mounting the female, the male's wings were in motion. Initial courtship contact between the sexes while both were in flight was rarely seen. The direction and angle of approach as well as the landing position of the male on the female was variable; therefore, the body parts of each sex which initially made contact during a given strike varied considerably. The male usually approached the female from the rear. But a male which did not land facing the same direction as the female would quickly reorient itself so that it

Figure 3-1. Male (on right) orienting to a tethered female.

faced the same direction as the female. Figure 3-2 shows a male striking from the rear of the female and Figure 3-3 shows a male striking from the side of the female. In these strikes the protarsi and mesotarsi touched the female before the metatarsi. However, in one video tape sequence, initial contact occurred when the male's metathoracic legs first touched and then grasped the female's abdomen.

When a male landed on the female, each of the female's wings became extended at approximately a 45° angle to her body. The male began to raise himself so that his body was above and parallel to the female's dorsum. The male moved forward until his head was over the female's thorax, then slightly raised his abdomen so that the angle between his body and the female's dorsum approached 45°. When the male was in this most forward position on the female, he used his prothoracic tarsi to rub the female's head (Figure 3-4). As he stopped rubbing the female's head and moved backward on the female, the male placed his prothoracic tarsi at the bases of the female's wings and his mesothoracic legs along the sides of the anterior portion of the female's abdomen. As the male continued to move backward, he slightly arched his abdomen downward past the tip of the female's abdomen and attempted to make genital contact (Figure 3-5) by grasping the slightly extended ovipositor of the female. Simultaneously, the male positioned his metathoracic legs firmly beneath the female's abdomen.

Figures 3-6 to 3-8 show several views of horn fly pairs *in copulo*. The male's prothoracic tarsi grasped the female at the wing bases; the metathoracic legs held the female laterally at the anterior of the female's abdomen; the metathoracic legs were held approximately parallel to each other beneath the female's abdomen. All of the female's legs

Figure 3-2. Male striking a tethered female from the rear of the female.

Figure 3-3. Male striking a tethered female from a position at the side of the female.

Figure 3-4. Male's prothoracic tarsi rubbing a tethered female's head when then the male was in the most forward position on the female's dorsum.

Figure 3-5. Male backed on the dorsum of a tethered female and attempting to copulate.

Figure 3-6. Horn flies *in copulo* (view A).

Figure 3-7. Horn flies *in copulo* (view B).

Figure 3-8. Horn flies *in copulo* (view C).

were in contact with the substrate. While in copulo, the pairs generally remained motionless except for some pairs in which the female occasionally walked about or cleaned herself with her metathoracic legs. If pairs in copulo were disturbed, the female with the male in position would briefly walk about or take flight. Males were not apparently distracted by this movement. Copulation usually was ended as the female became more active and made movements such as rapid wing vibration or kicking with the metathoracic legs to dislodge the male (Figure 3-9).

The movement of the male's wings continued throughout most of the courtship sequence until the male was in position to make a copulatory attempt. Males unable to copulate on an initial attempt often repeated earlier elements of the courtship sequence while on the female's dorsum. The male would often move forward again, rub the female's head and back while positioning himself for another copulatory attempt. Other males would stop their wing movement and rest motionless on the female's dorsum (Figure 3-10) until either resuming courtship or flying off. Other males would back off of the female and remain motionless about 1 fly length from the female. Some males would remain in this position facing the female's abdomen for up to several minutes before resuming courtship or flying off. Other males behaved similarly but kept one or two of their prolegs on the female's abdomen or wings (Figure 3-11). This resting behavior on or near the female was designated arrested movement.

Tethering the female versus having the female free to move about appeared to increase the frequency of arrested movement (++) shown by the male. However, the arrested movement of the male on the female

Figure 3-9. Female dislodging a male at the termination of copulation.

Figure 3-10. Male which had arrested its movement on a tethered female's dorsum.

Figure 3-11. Male which had arrested its movement after an unsuccessful copulatory attempt with a tethered female. The male had backed off of the female's dorsum but had kept its prothoracic legs on the female's wings.

was also seen during the interaction of pairs in the laboratory rearing cages in which both sexes were free to move about. Although males were often prevented from actively continuing courtship because of the female's resting position or defensive behavior, some males were observed to rest on the female's dorsum for approximately 30 seconds to 1 minute before either flying off or resuming courtship. In addition, the qualitative comparison of data from several experiments suggested that when virgin females were tethered they were less likely to copulate in a given period of time than were virgin, untethered females. The frequency of copulation will be discussed for these experiments of concern.

Whether tethered or untethered, females displayed considerable defensive behavior. The advances of sexually aggressive males were often thwarted by the female's choice of a relatively protected resting position or by her defensive movements. In the observation container, the photographic arena, or rearing cage, untethered females often rested along the inside edges of the cage, particularly the top edge, which prevented strikes of males from behind and appeared to minimize the females' contact with males. A female could also stop courtship activity by 1) curling her abdomen downward while holding her overlapped wings high above her body; 2) kicking with her legs, especially the metathoracic legs (Figure 3-12); 3) rapidly vibrating her wings; 4) flying or moving away from the male; or 5) by turning their body to put the male at her side. Each of these defensive movements could be used singly or in combination preventing the male from effectively mounting the female or effectively positioning himself on the female to make a copulatory attempt.

Figure 3-12. Tethered female kicking at the male with one of her metathoracic legs.

Male interaction with other males was occasionally observed. Males would strike other untethered males in the observation container but would rarely strike a tethered male. Males would also readily strike the dorsum of a male already courting a tethered female (Figure 3-13) forming a stack of males upon a tethered female (Figure 3-14). On a number of occasions groups of males would cluster about a tethered female with each male attempting to grasp the female (Figure 3-15); usually this resulted in no male being able to effectively court the female. At other times, males did not cluster about the tethered female, but actively competed for the female (Figure 3-16). Often 1 male could thwart or block the attempts of other males to dislodge him from his position on the female. But at other times, males were successful in dislodging another male off of the female's dorsum.

Courtship Behavior of Untethered Pairs of Males and Females

The observation of courting pairs of 4-6 day old, virgin horn flies permitted the quantification of several parameters of courtship (Tables 3-1, 3-2, A-2). Considerable variability existed among pairs in the time required from when the pairs were placed together until a given element of the courtship interaction occurred. The mean time (n = 19) between placing the pair together until the first strike occurred was 7 min 47 sec (Table 3-1), but individual values ranged from 15 sec to 30 min 2 sec. Likewise, the time from the first strike until copulation began ranged from 12 sec to 25 min with a mean of 6 min 52 sec. The total time from placing the pairs together to the beginning of copulation ranged from 2 min to 33 min 30 sec with a mean

Figure 3-13. Second male striking the dorsum of a male already courting a tethered female.

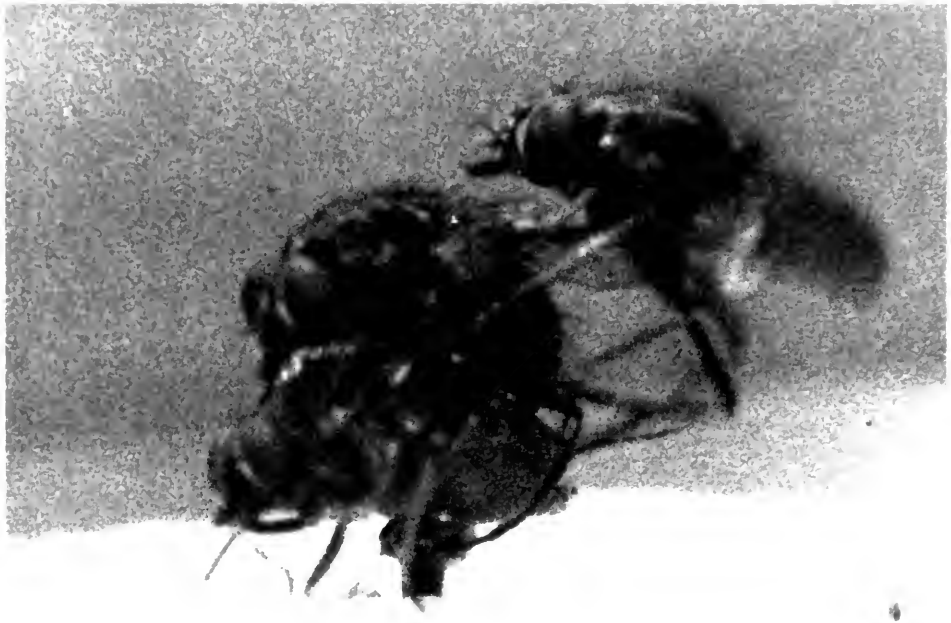


Figure 3-14. Two males in a stack upon the dorsum of a tethered female.

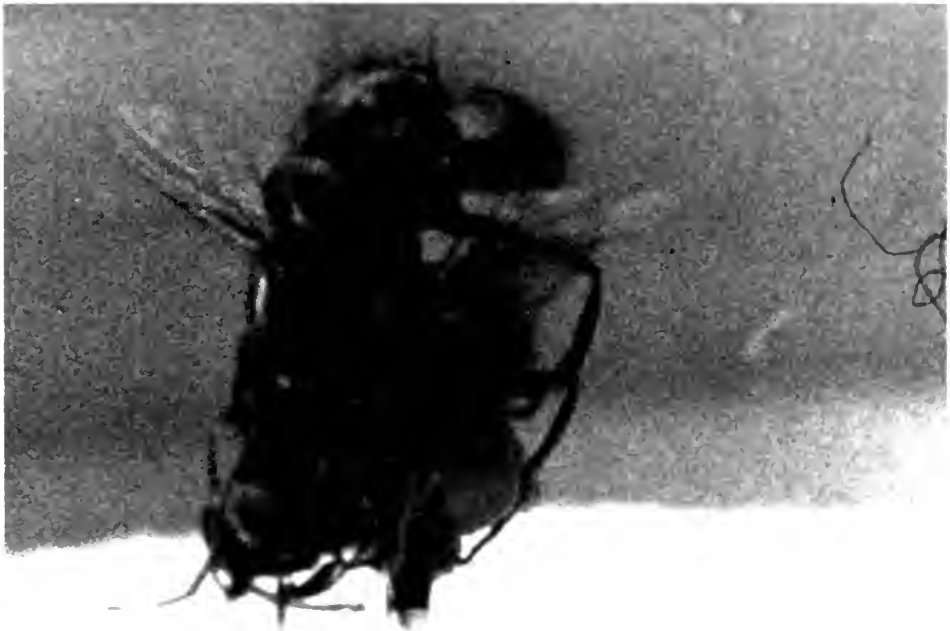


Figure 3-15. A group of males clustered about a tethered female.

Figure 3-16. Two males striking a tethered female.

Table 3-1. Courtship parameters for untethered virgin male and virgin female pairs.[a]

Parameter[b]	$\bar{X}$	Median	Range
Start to first strike	7:47	5:06	0:15-30:02
Courtship duration (First strike to beginning of copulation)	6:52	2:59	0:12-25:00
Start to beginning of copulation	14:32	11:41	2:00-33:30
Copulation duration	6:14	6:01	3:24-11:23

[a]Nineteen pairs of flies were tested; flies were 4-6 days old and had been reared individually from emergence. Raw data are listed in Table A-2.

[b]Parameters expressed as min:sec.

Table 3-2. Courtship behavior of untethered virgin pairs[a] of H. irritans.

Pair No.	(+) Strike	(++) Arrested movement	(+++) Positioning	(++++) Copulation
1	16	10	1	1
2	22	3	2	1
3	1	1	1	1
4	3	1	1	1
5	1	1	1	1
6	3	1	1	1
7	3	1	1	1
8	1	1	1	1
9	4	1	1	1
10	8	1	1	1
11[b]	13	0	0	0

[a]Both males and females were 5 days old and had been reared individually from emergence; 11 pairs were observed.

[b]The female of this pair was unreceptive. Observation of the pair lasted 1 hr without copulation occurring. After the first male was removed, a second virgin male was placed with this female. After 24 hr, examination of the female's spermatheca revealed that no sperm were present.

of 14 min 32 sec. The mean copulation duration was 6 min 14 sec with a range of 3 min 24 sec to 11 min 23 sec. The courtship data for each pair in Table A-2 reflect that individual pairs interacted at different rates. Some males, such as male No. 15, rapidly struck the female when placed in the observation container, but courted for several additional minutes before copulation began. Other males, for example male No. 17, took longer before initially striking the female, yet were able to initiate copulation quickly. Between these 2 extremes were the majority of males which had intermediate times.

In about half of the trials, males would quickly strike the female with which they had just copulated. Females generally displayed distinct defensive behavior to thwart these advances. However, females No. 1 and No. 3 copulated for 4 min 33 sec and 4 min 10 sec, respectively, within 1 min of the completion of their first copulation (Table A-2). Examination for sperm transfer in all females used in these trials indicated that only female No. 6 had apparently not obtained sperm during what had appeared to be a normal copulation. In all other females sperm was present in the spermatheca. The courtship activity data listed in Table 3-2 also indicated that the courtship activity among individual pairs (n = 11) was variable. For example, with pair No. 1 and No. 2 considerable courtship activity occurred between the sexes before copulation began; however, in pairs No. 3, No. 5, and No. 8 copulation occurred after the first strike. Pair No. 11 did not copulate; the female of the pair was unreceptive to the male for 1 hr and was apparently unreceptive to a second male which was substituted for the first male after 1 hr. Examination of this female's spermatheca after she had been held with the second male for 24 hrs indicated that sperm had not been transferred.

Courtship Behavior of Tethered Horn Flies

The behavioral responses of virgin male horn flies to tethered virgin male and female horn flies are presented in Table 3-3. The number of touches varied significantly from period to period, but the number of touches generally increased with each consecutive period. T tests comparing the means of each sex for each period indicated that only in the second period did males touch females significantly more ($P < .05$) than they touched males; females were touched 1.9x more than were males. In the other 3 periods, no differences were apparent.

Although the data did not clearly demonstrate that males touch females more frequently than they touch males, a highly significant difference ($P \leq .0001$) was indicated in the number of strikes received by females and males. Males struck females an average of 14.8x more than they struck males. Also, males positioned themselves on the dorsum of the female significantly more ($P \leq .0414$) than they did upon males. During 4 hours of observation, only once did a male position itself on the dorsum of a tethered male. Males made no copulatory attempts upon other males, but made significantly more copulatory attempts upon females ($P < .10$); however, in 4 hours of testing time with groups of 35 males and 6 females, only 3 copulations were observed. Thus, tethered females appeared less likely to mate than did untethered females (Tables 3-2 and A-2).

The analysis of the mean percentage continuation for each sex indicated that males proceeded through each element of the courtship sequence more often with females than with males; males proceeded from a touch to a strike ($P \leq .0001$), from a strike to positioning on the

Table 3-3. Behavioral responses of virgin male horn flies to tethered virgin male and female horn flies and percentages of behaviors which were followed by the next behavior in the courtship sequence.[a]

	Mean Number of Responses/min[b]			
	Touch	Strike	Positioning on dorsum	Copulatory[c] attempt
Male	8.80	0.43a	0.00a	0.00a
Female	13.48	6.38b	0.36b	0.13b

	Mean Percentage Continuation					
	Touch	→	Strike	→	Positioning on dorsum	→ Copulatory attempt
Male	5.2a		0.4a		0.0a	
Female	49.1b		6.3b		34.0b	

[a]Data based on 16, 15-minute periods per sex tested with 35 virgin males and 6 tethered flies per period. All flies were 5-6 days old.

[b]Means of the same vertical column not followed by the same letter are significantly different at at least $P < 0.10$ as determined by appropriate F tests.

[c]During these tests a total of only 3 copulations were observed.

dorsum ($P \leq .0711$), and from positioning on the dorsum to a copulatory attempt ($P \leq .0251$) significantly more with the females than with males. Males which touched males rarely continued to court that male. Males infrequently struck other males, rarely positioned on another male's dorsum, and never were observed attempting to copulate with a tethered male. However, when males courted females almost 50% of the touches continued to strikes. While only about 6% of the strikes by males led to successful positioning on the dorsum of the female, once positioning was accomplished approximately 34% of these positioning movements led to actual copulatory attempts.

The behavioral responses of virgin and mated males of different age categories to tethered virgin and mated females of the same age categories are summarized in Tables 3-4 and 3-5. No comparisons in the number of touches among the 5 treatment combinations were made due to a significant period to period effect in the analysis of variance; however, no differences among treatment combinations were evident for strikes, positioning on the dorsum, or copulatory attempts. Although no analysis was made on the number of copulations observed for each treatment combination, the total number of copulations observed in each 2 hour observation of each treatment is listed in Table 3-4.

The analysis of the mean percentage continuation for each treatment indicated that no apparent differences were evident between treatment combinations for the percentage of touches continuing to strikes; however, the continuation of strikes to positioning on the dorsum was dependent on the mating status of the male and female. Mated males and females continued this portion of the courtship sequence more often than did pairs of virgin males and females. No differences among

Table 3-4. Behavioral responses of virgin and "mated"[a] male horn flies of different ages to tethered virgin and "mated" female horn flies of different ages.[b]

Status/Age	Mean Number of Responses/min[c]				
Males x Tethered Females	Touch	Strike	Positioning on Dorsum	Copulatory Attempt	Total No. Copulations
mated (8-day) x mated (8-day)	13.1	3.0a	0.3a	0.1a	2
virgin (3-day) x mated (9-day)	19.6	6.4a	0.4a	0.1a	4
mated (8-day) x virgin (3-day)	15.1	3.6a	0.4a	0.2a	5
virgin (3-day) x virgin (3-day)	20.8	7.7a	0.2a	0.0a	1
virgin (8-day) x virgin (8-day)	24.6	5.1a	0.1a	0.1a	3

[a]Held with flies of the opposite sex, therefore, considered to be mated.

[b]Data based on 8, 15-minute periods per treatment combination with 35 males and 6 tethered females per period.

[c]Means of the same vertical column not followed by the same letter are significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

Table 3-5. Behavioral responses of virgin and "mated"[a] male horn flies of different ages to tethered virgin and "mated" female horn flies of different ages.[b]

Mating status/Age	Mean Percentage Continuation[c]			
Males x Tethered Females	Touch →	Strike →	Positioning on Dorsum →	Copulatory Attempt
mated x mated (8-day) (8-day)	24.3a	10.4a	37.9a	
virgin x mated (3-day) (9-day)	42.0a	6.7ab	11.1a	
mated x virgin (8-day) (3-day)	27.2a	9.6ab	51.5a	
virgin x virgin (3-day) (3-day)	37.9a	3.4b	3.1a	
virgin x virgin (8-day) (8-day)	23.6a	3.6b	35.8a	

[a]Held with flies of the opposite sex, therefore, considered to be mated.

[b]Data based on 8, 15-minute periods per treatment combination with 35 males and 6 tethered females per period.

[c]Means of the same vertical column not followed by the same letter are significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

treatment combinations were noted for the mean percentage continuation from positioning on the dorsum to copulatory attempts.

The hierarchy of courtship behavior of virgin male horn flies to different treatments of tethered males and females is summarized in Table 3-6. The number of males responding to all female treatments when the female treatments data were pooled was significantly greater than the number of males responding to all male treatments when pooled (X^2 = 163.36, P < .0001); however, for each sex no significant variation among treatments for that sex was noted (males, X^2 = 2.18, N.S.; females, X^2 = 2.70, N.S.). Thus, the sex of the fly affected the number of responding males more than did the treatment that the fly received. All treatments of males received few courtship advances by males; in addition, the hierarchy of the observed courtship responses was similar among the 4 treatment groups involving males. However, the hierarchy of response to each of the female treatments was not similar as the dead, winged female treatment received more copulations than did any other treatment (Figure 3-17). Specifically, the dead, winged females received copulations in about 33% of the trials while the live, winged females received no copulations. The hierarchy of response to the dead, winged female treatment was significantly different than the response hierarchy to the live, winged female treatment (X^2 = 9.81, P < .05).

In another series of tests the hierarchy of response of 1 male versus 5 males to tethered, virgin males and females was compared (Table 3-7). Regardless of whether 1 male or 5 males were in the test container with the tethered fly, live tethered males rarely received any courtship advances while live females often received courtship

Table 3-6. Hierarchy of courtship behavior of virgin male horn flies to different treatments of tethered males and females.[a]

Treatment	No. responding	Frequency of Response			
		(+) Strike	(++) Arrested movement	(+++) Positioning on Dorsum	(++++) Copulation
Live female (winged)	27	10	15	2	0
Live female (wingless)	29	6	22	1	0
Dead female[b] (winged)	28	8	10	1	9
Dead female (wingless)	26	14	11	0	1
Live male (winged)	2	1	1	0	0
Live male (wingless)	2	2	0	0	0
Dead male (winged)	2	2	0	0	0
Dead male (wingless)	5	5	0	0	0

[a]All flies were virgin and 3-6 days old.

[b]Males and females were killed by freezing at -15 C and thawed before being tested.

[c]For each treatment, 30, 3-minute trials were conducted with 5 virgin males used per trial.

Figure 3-17. Male copulating with a tethered, dead, winged female.

Table 3-7. Comparative hierarchy of courtship behavior of groups of 1 or 5 virgin males to tethered virgin males and females.[a]

Treatment	No. Trials	No. Trials with Responding	Frequency of Response			Time to First Strike $\bar{X} \pm$ S.E. (sec)
			+ (Strike)	++ (Arrested movement)	+++ (Positioning on dorsum)	
1 male per container						
Live female	46	22	5	12	5	54.1 ± 44.7[c]
Live male	46	1	1	0	0	1.4 ± 0
5 males per container						
Live female	30	24	8	8	8	62.5 ± 57.8[c]
Live male	30	0	0	0	0	0

[a]All flies were 3-8 days old.

[b]Each trial was 3 minutes.

[c]The T test between the live female means was insignificant.

advances. The number of males in the test container did not affect the time required before the female received the first strike; a T test comparing the 2 treatment means was insignificant ($P > .05$); however, the number of tests in which males responded to females during each 3-minute test was affected by the number of males per container. With only 1 male per container, courtship behavior was only observed in 47.8% of the trials, but with 5 males per container, courtship behavior was seen in 80.0% of the trials. This difference was significant ($\chi^2 = 7.87$, $P < .025$).

Discussion

The preliminary observation of horn fly behavior under laboratory rearing conditions confirmed that in the Florida Laboratory Strain, sexual activity did not begin until about 2.5 days after emergence; this finding agreed with the data of Harris et al. (1968). The general courtship behavior of mature, well-nourished H. irritans males and females was similar to that described for other muscid species (Anderson, 1978; Colwell and Shorey, 1975; Tobin and Stoffolano, 1973a and b; Tauber, 1968). Perhaps the most distinct element of the horn fly courtship sequence noted in these studies was the rubbing of the female's head by the male's protarsi before the male backed on the female and attempted to copulate. No manipulation of the female's prolegs was observed as was seen in house fly courtship by Colwell and Shorey (1975). The point of first contact between the sexes and landing position of the male were variable and influenced by the angle of approach of the male as has been reported for the house fly by Colwell and Shorey (1975).

The amount of courtship preceding copulation and the frequency of performed courtship elements was variable for untethered pairs. The duration of copulation was also variable from pair to pair with a range of 3 min 24 sec to 11 min 23 sec versus the range reported by Bruce (1964) of 30 sec to 5 min for pairs which copulated on the host. The present experiments also indicated that some females may remate; Harris et al. (1968) had reported that the female horn fly was monogamous.

Non-receptivity of the female resembled that described for other muscid females; however, the female horn fly's self-positioning in locations in the observation cage which appeared to reduce her contact with males had not been noted for other muscid females. As in house fly courtship, males encountering an unreceptive female may leave her or may move forward on her dorsum and repeat earlier courtship elements. The arrested movement of horn fly males on or near females may be related to the guarding of females by males or the pre-copulatory passive phase discussed by Parker (1970c and 1974). In the horn fly as in Musca domestica (L.) (Colwell and Shorey, 1975) and Fannia femoralis Stein (Tauber, 1968) the female appears to control the termination of copulation.

Experiments with tethered males and females demonstrated that males would initiate courtship and continue to court tethered females with greater frequency than they would with tethered males. The mean number of various courtship responses of males to females per unit time differed somewhat from similarly conducted studies with house flies (Colwell and Shorey, 1975). Horn fly females received about 10x more touches and about 6x more strikes per unit time than did house fly females. In addition, about 10x fewer copulatory attempts per unit time were received

by horn fly females than were reported for house fly females. However, as is the case in house fly courtship, mated and virgin females were equally courted by either virgin or mated males.

Tethering the female may have affected the female's receptivity. Qualitative comparisons between the number of copulations measured for different tests where females were either tethered or untethered (Tables 3-4 and A-2) indicated that tethered females were less likely to copulate within the period of time that untethered females normally began to copulate. Colwell and Shorey (1975) had noted that tethered house fly females displayed defensive behavior that Tobin and Stoffalano (1973a) previously had reported to be normal courtship behavior.

Female wing positioning by horn fly females does not appear to be as important in horn fly courtship as in stable fly courtship (Anderson, 1978). In fact, horn fly males readily courted wingless, live or dead females (Table 3-6). As is the case in house fly courtship (Colwell and Shorey, 1975) dead females with or without wings were courted more than were dead males with or without wings. Also, as was noted in those house fly studies, dead, wingless horn fly females received about the same number of copulatory attempts as did live, winged females. But contrary to the house fly studies by Colwell and Shorey (1975), dead, winged female horn flies received more copulations than did live, winged females. However, dead, wingless females received few copulations. When the wings were removed from a dead female, males may not have been able to properly position themselves for a copulatory attempt. Unlike Fannia femoralis males, horn fly males readily courted still females. The copulation of horn fly males with dead females indicated that movement of the female was not a prerequisite for courtship behavior.

The means by which males differentiated males from females during the early elements of courtship were not clarified by these studies. The difference between males and females, however, could still be determined by courting males whether the males and females were live, dead, winged, or wingless. Genetic, physiological, and environmental variables which may affect horn fly courtship remain to be investigated.

CHAPTER 4

A MATING STIMULANT PHEROMONE OF THE HORN FLY, *Haematobia irritans* (L.): DEMONSTRATION OF BIOLOGICAL ACTIVITY IN SEPARATED CUTICULAR COMPONENTS

Abstract

Preliminary bioassays on the horn fly, *Haematobia irritans* (L.), suggested that a mating stimulant pheromone was involved in horn fly courtship behavior. Virgin males readily courted live or dead females but rarely made mating strikes upon live males, dead males, or females thoroughly washed with hexane. Bioassays utilizing the response of virgin males to treated, virgin male horn flies indicated that female cuticular hydrocarbons were responsible for inducing male courtship behavior. Specifically, the female paraffin and monoolefin fractions were biologically active when bioassayed alone or in combination. Three synthetic monoolefins previously shown to be the major components in the female monoolefin fraction were biologically active in bioassays. The compounds (*Z*)-5-tricosene, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene were each active, but greater male courtship behavior was observed when these 3 compounds were bioassayed in combination.

Introduction

The horn fly, *Haematobia irritans* (L.), rapidly became an important pest of livestock in the United States after its introduction about 1887 around Camden, New Jersey (Riley, 1889). Estimated losses of over

$179 million dollars to the livestock industry occur annually as the result of fly annoyance on animal weight gains and milk production (Graham and Hourrigan, 1977; Knipling, 1972). Recent integrated control strategies with the objective of horn fly eradication have utilized insecticide sprays, sterile male release, and the insect growth regulator methoprene (Graham and Hourrigan, 1977; Kunz, 1978).

A number of sex pheromones have been identified for flies of the family Muscidae. A sex attractant, (Z)-9-tricosene, for male house flies, Musca domestica L., caused both male and female house fly aggregation in the field (Carlson et al., 1971; Carlson and Beroza, 1973). Male mating stimulant compounds have been identified for 6 other species of muscoid flies. The sex stimulant pheromone of the stable fly, Stomoxys calcitrans (L.), which induced male copulatory behavior at short range has been described as a complex of methyl-branched paraffins and monoolefins which were most active in combination. To date, this complex is known to include (Z)-9-hentriacontene, (Z)-9-tritriacontene, 13-methyl-1-hentriacontene, 13-methyl-1-tritriacontene plus an array of methyl-branched hentriacontanes and methyl-branched tritriacontanes in which 11- and 15-methyl-substituted alkanes are the most active (Muhammed et al., 1975; Uebel et al., 1975b; Sonnet et al., 1977; Sonnet et al., 1979). Long chain olefins [(Z)-14-nonacosene, (Z)-13-nonacosene, and (Z)-13-heptacosene] identified from the face fly, Musca autumnalis De Geer, stimulated male courtship behavior; nonacosane and heptacosane attenuated the olefins' activity (Uebel et al., 1975a; Sonnet et al., 1975). (Z)-9-pentacosene has been identified as the mating stimulant pheromone for males of Fannia canicularis (L.) (Uebel et al., 1977) while (Z)-11-hentriacontene was active in

stimulating courtship responses of F. pusio and F. femoralis males (Uebel et al., 1978a and 1978b). Long chain di- and trimethyl-branched paraffins have been identified as releasers of male mating behavior in the tsetse fly, Glossina morsitans morsitans Westwood (Langley et al., 1975; Carlson et al., 1978).

Mackley (1977) demonstrated that horn fly courtship was, in part, chemically mediated; male horn flies, H. irritans, made a significantly greater number of prolonged contacts with live or dead females than with live males or females which had been thoroughly washed in hexane. Olfactometer studies also indicated a slight attraction of both males and females to the female hydrocarbon fractions. Four major monoolefins, (Z)-9-tricosene, (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene, were identified and obtained as synthesized compounds. Laboratory males contained a relatively greater amount of (Z)-9-tricosene, but laboratory females had relatively greater amounts of the other three monoolefins. Bioassays for mating stimulant activity of these compounds were unsuccessful because of a lack of response by males to fly models (Mackley, 1977).

Preliminary observation of the courtship sequence of horn flies (Chapter 3) suggested a suitable bioassay which was used to investigate cuticular components for pheromone activity.

Materials and Methods

Horn flies used in these studies were from the laboratory colony maintained at the University of Florida (Florida Laboratory Strain, F) as described by Greer (1975). Adults were kept in aluminum-frame,

screened cages (25.5 x 24 x 15 cm) or in 140 ml clear plastic specimen containers which were fitted with screen tops. Within 24 hours of eclosion, male and female horn flies to be used in bioassays or for extract preparation were immobilized by carbon dioxide anesthesia, sexed, and reared separately until needed. Adult rearing was at a temperature of 27 ± 2°C and a relative humidity of 70 ± 10%; continuous lighting for the colony was provided by 6 Westinghouse F40CW (40 W) Cool White[3] fluorescent bulbs. Adults could obtain blood meals at will through the cage top from gauze-covered pads of absorbant surgical cellulose (Doeskin Products, Inc., New York, NY) soaked with bovine blood treated with 0.1 gm of Kanamycin[4], 30,000 units of Mycostatin[5], and 3.75 gm of sodium citrate per liter. Blood pads on adult cages were changed twice daily.

Bioassays were conducted when virgin, laboratory horn flies were 3-8 days old since the data of Harris et al. (1968), Gale (1977), and Mackley (1977) indicated that flies of that age would be sexually active. Candidate lipid extracts, fractions, or synthetic compounds were applied to virgin, male horn flies designated as T males. After anesthetizing each T male with carbon dioxide, the ventral surface of its abdomen was glued to the head of an insect pin with Weldwood Contact Cement[6] (U.S. Plywood, Kalamazoo, MI). Live males and females used in some tests were similarly prepared and were designated as "tethered." After T males were killed with a brief exposure to -15°C temperature, compounds to be bioassayed were applied by a syringe to the dorsum of the abdomen and thorax. The insect pin was inserted through an 8 mm thick, white polyethylene foam sheet (Bernel Foam Products Co., Inc., Buffalo, NY) so that the excess pin beneath the foam sheet could be cut

off allowing the T male to be in position approximating a normal standing position. The foam sheet with the T male in place was set upon a second sheet of polyethylene foam position on a Plexiglas® platform. Three centimeters below the Plexiglas® platform was a 22 watt Westinghouse Cool White® circle lamp (21.5 cm diameter); the illumination at the foam surface below the T males was approximately 25 foot candles.

Five 3-8 day old virgin males were transferred into a screened-topped, clear plastic specimen container through a circular hole in the bottom of the container. In one series of bioassays only one male was placed in the container per trial (Table 4-2). The frequency of response to treatments was evaluated by observing if courtship behavior was displayed toward the T male within a 3-minute period after the specimen container holding the virgin males was placed over the T male. The first observed courtship behavior was scored as follows: 1) strike (+), an attempt by the virgin male to get onto the dorsum of the T male; 2) arrested movement (++), after striking the T male, the responding male maintained contact with the T male; 3) positioning (+++), after striking the T male, the responding male made characteristic grasping movements with its legs around the T male and positioned its genitalia below the terminal portion of the T male's abdomen. One point was arbitrarily assigned for each level of this hierarchy of male courtship behavior. Thus, the first responding male could earn a maximum response score of 3 points. If no courtship behavior was observed within the 3-minute period, a score of nil was recorded. For each extract, fraction, or compound tested, a minimum of thirty 3-minute trials were performed with a maximum possible response score of 90. Controls and treatments were bioassayed in a completely random order

within a given series of trials. All virgin males were discarded after one test.

The described bioassay was based on initial observations of the hierarchy of courtship behavior that occurs between virgin males and tethered males and females in the laboratory. In each series of bioassays, the response of virgin males to both live females and hexane-treated T males (or live males) was evaluated. These controls provided a continuous measure of the general responsiveness of test insects. Virgin males usually courted tethered females (+, ++, or +++), but rarely mated with them. However, males rarely made strikes (+) and virtually never displayed further sexual behavior (++ or +++) toward tethered males.

The frequency data from bioassays were analyzed utilizing the chi-square test for 2 x K contingency tables (Steel and Torrie, 1960; Ostle, 1963). The biological activity of an extract, fraction, or synthetic compound was evaluated by comparing the number of males responding to the treated T males versus the number of males responding to hexane-treated T males in the same series of bioassays. The hierarchy of male response to biologically active treatments was compared to the hierarchy of response to live females in the same series of bioassays.

Crude lipid extracts were obtained from 3-4 day old males and females of the Florida Laboratory Strain (F). These flies were lightly anesthetized with carbon dioxide and rinsed with a volume of hexane equivalent to 4 ml per fly. After the extract was filtered through a Whatman No. 4 filter, the hexane was removed under vacuum in a rotary evaporatory and by evaporation with a stream of nitrogen.

Female crude lipid extracts were fractionated by liquid chromatography on 1 cm ID x 45 cm columns of activated silica gel (60-200 mesh, J.T. Baker Chemical Co., Phillipsburg, NJ). The hydrocarbon fraction was eluted with 50 ml of hexane. More polar fractions were eluted with additional 50 ml volumes of hexane with increasing quantities of ether (5%, 10%, 50%). Separation of hydrocarbons from other lipid classes was confirmed by thin layer chromatography (TLC) on activated silica gel plates (250 μ Anasil, Analabs, Inc., North Haven, CT) developed with a hexane:ether:acetic acid (90:10:1 v/v) solvent mixture.

The hydrocarbon fraction was separated into fractions by liquid chromatography on 1 cm ID x 45 cm columns packed with 20% silver nitrate impregnated silica gel (60-200 mesh, Hi-Flosil-Ag, Applied Science Laboratories Inc., State College, PA). The paraffins were eluted in the first fraction with 50 ml of hexane; the monoolefins were eluted in the fourth fraction with a 50 ml volume of 5% ether in hexane. Separation of fractions was confirmed by TLC on silica gel plates impregnated with 20% silver nitrate (250 μ Uniplates, Analtech Inc., Newark, DE) developed with a hexane:benzene (75:25 v/v) solvent mixture. All hexane used in these investigations was stirred over concentrated sulfuric acid for two days, washed with distilled water, distilled over sodium, and stored over sodium hydroxide. Synthetic monoolefins bioassayed were those whose identification and synthesis have been described by Mackley (1977).

Results

The existence of a male mating stimulant pheromone in the horn fly was suggested in preliminary bioassays since mature virgin males readily

initiated courtship behavior with mature, live or recently dead females but initiated few strikes upon females which had been thoroughly rinsed 10x with 4 ml volumes of hexane (Table 4-1). The number of males responding to live females (x^2 = 38.40, $P < 0.001$) and dead females (x^2 = 48.65, $P < 0.001$) was significantly greater than the response to live males. The number of males responding to hexane-washed females was low and not significantly different than the response to live male controls (x^2 = 1.07, N.S.). The few responses of males toward hexane-washed females consisted only of strikes (+). However, males displayed both (++) and (+++) behavior to live and recently dead females giving response scores of 55 and 65, respectively, for these females. When a 2 x 4 contingency table analysis was made comparing the frequencies of male responses [no response, (+), (++), and (+++)] to live and recently dead females, the x^2 value was not significant (x^2 = 4.03, N.S.). We view this x^2 test as an index indicating that the hierarchy of male response to live and recently dead females is similar.

Throughout these bioassays, males were rarely observed striking live, tethered males, recently dead males (Table 4-1), or hexane-treated T males (Tables 4-2 thru 4-8). Also, no (++) or (+++) behavior was observed between these males. On occasion, live males in the bioassay container did make strikes upon other untethered virgin males in the container, but these strikes were not recorded.

A second bioassay series (Table 4-2) demonstrated that a significantly greater number of males responded to 2 fly equivalents of female crude extract than to hexane-treated T males (x^2 = 7.92, $P < 0.005$). When the total hydrocarbon and total non-hydrocarbon fractions from the female were bioassayed at 2 fly equivalents each, activity was found

Table 4-1. Hierarchy of courtship behavior of adult male H. irritans to live and treated virgin male and female horn flies.

Treatment[a]	No ♂♂ Responding	Frequency of Response[c]			Response[d] Score (max. = 90)
		+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	27*	4	18	5	55
Live male (control)	1	1	0	0	1
Hexane-washed female	3	3	0	0	3
Dead female[b]	28*	3	13	12	65
Dead male[b]	1	1	0	0	1

*$P < 0.001$.

[a]Each treatment tested in 30, 3-minute trials.

[b]Killed by freezing at -15 C and thawed before being bioassayed.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Table 4-2. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected lipid extracts.

Treatment	Amount	No. Responding	Frequency of Response[c]			Response[d] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	14**	3	7	4	29
Live male (control)	--	1	1	0	0	1
Hexane-treated T male (control)	1.0 µl	0	0	0	0	0
Female crude lipid extract	2 F.E.[b]	7*	4	2	1	9
Female total hydrocarbons	2 F.E.	12**	5	3	4	21
Female total non-hydrocarbons	2 F.E.	1	1	0	0	1

*P < 0.005.

**P < 0.001.

[a]Each treatment tested in 30, 3-minute trials; one adult male was tested per trial.

[b]F.E., fly equivalents.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

to reside in the hydrocarbon fraction; the response rate of 40% of the males to the total hydrocarbon fraction was significantly greater than the response to hexane-treated T males (x^2 = 15.00, $P < 0.001$). Male response to T males treated with 2 fly equivalents of the female total non-hydrocarbons was not significantly different than the response to hexane-treated T males. The response score of 21 for the female total hydrocarbons was the closest to the live females' score of 29. The hierarchy of response to live females and the female total hydrocarbons were similar as indicated by the contingency table analysis (x^2 = 2.22, N.S.).

When the individual female hydrocarbon fractions were bioassayed at 4 fly equivalents each (Table 4-3), a significantly greater number of virgin males responded to both the female paraffins (fraction 1) (x^2 = 15.02, $P < 0.001$) and the female monoolefins (fraction 4) (x^2 = 22.26, $P < 0.001$) than to hexane-treated T males. The male responses to the paraffin and monoolefin fractions were not significantly different from each other (x^2 = 1.07, N.S.) on the basis of the number of responding males; however, males displayed (+++) behavior toward the T males treated with monoolefins but not toward T males treated with paraffins, giving a response score for the monoolefin fraction of 34 versus 19 for the paraffin fraction. Nevertheless, the hierarchy of response to the live female was significantly different (x^2 = 8.57, $P < 0.05$) than the hierarchy of response to the female monoolefin in fraction. This series of bioassays also demonstrated that the male crude lipid extract was not biologically active.

To determine if a combination of active fractions would increase male response, the paraffin and monoolefin fractions were bioassayed

Table 4-3. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected lipid fractions.

Treatment[a]	Amount	No. ♂♂ Responding	Frequency of Response[c]			Response[d] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	27*	8	10	9	55
Live male (control)	--	0	0	0	0	0
Hexane-treated T male (control)	2 µl	1	1	0	0	1
Female hydrocarbon						
Fraction 1	4 F.E.[b]	14*	9	5	0	19
Fraction 3	4 F.E.	3	3	0	0	3
Fraction 4	4 F.E.	18*	8	4	6	34
Male crude lipid extract	4 F.E.	0	0	0	0	0

*$P < 0.001$.

[a]Each treatment tested in 30, 3-minute trials.

[b]F.E., fly equivalents.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

singly and in combination (Table 4-4). At doses of 4 fly equivalents per T male, the paraffin fraction, monoolefin fraction, and paraffin/monoolefin combination (1:1 mixture) each gave a significantly greater number of male responses than did hexane-treated T males, but the combination of paraffins and monoolefins did not produce a greater number of responding males than did the monoolefin fraction alone. Thus, at the doses tested, the natural fractions did not appear to be additive in stimulating male response.

Several series of bioassays were conducted with synthetic monoolefins which previously have been identified as the four major monoolefins in the female cuticular hydrocarbons (Mackley, 1977). Two mixtures of these compounds, formulated at ratios as they occur in the natural female monoolefin fraction, were tested at 10 µg and 40 µg per T male (Table 4-5). At 10 µg, the response to each mixture was not significantly different than the response to the hexane-treated T male, but both mixtures were significantly more active at 40 µg than were T male controls. The number of males responding to the (Z)-5-tricosene (Z)-9-tricosene mixture (73:27) at 40 µg was significantly different than T male controls ($x^2 = 10.76$, $P < 0.005$) while the combination of (Z)-5-tricosene, (Z)-9-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene (62:23:9:5 mixture) at 40 µg evoked a greater male response ($x^2 = 17.33$, $P < 0.001$). In addition, this later combination received the highest response score (20) in this series of bioassays except for the live female (37). When tested singly, (Z)-9-pentacosene and (Z)-9-heptacosene treated T males were significantly more active in inducing male responses than were hexane-treated T males (Table 4-6) ($x^2 = 14.70$, $P < 0.001$ and $x^2 = 7.20$, $P < 0.01$, respectively). The total response

Table 4-4. Hierarchy of courtship behavior of adult male H. irritans to T males treated with hydrocarbon fractions.

Treatment[a]	Amount	No. of Responding	Frequency of Response[c]			Response[d] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	30	10	15	5	55
Hexane treated T male (control)	2 µl	4	4	0	0	4
Paraffin fraction	4 F.E.[b]	11*	11	0	0	11
Monoolefin fraction	4 F.E.	17**	16	1	0	18
Paraffin and monoolefin fractions (1:1 mixture)	4 F.E.	17**	15	2	0	19

*$P < 0.05$.

**$P < 0.001$.

[a]Each treatment tested in 30, 3-minute trials.

[b]F.E., fly equivalents.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Table 4-5. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected synthetic monoolefins.

Treatment[a]	Amount	No. Responding	Frequency of Response[b]			Response[c] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	26**	15	11	0	37
Hexane-treated T male (control)	2 µl	2	2	0	0	2
(Z)-5-tricosene	a) 10 µg	7	7	0	0	7
(Z)-9-tricosene (73:27 mixture)	b) 40 µg	13*	13	0	0	13
(Z)-5-tricosene	a) 10 µg	5	5	0	0	5
(Z)-9-tricosene (Z)-9-pentacosene (Z)-9-heptacosene (62:23:9:5 mixture)	b) 40 µg	17**	14	3	0	20

*P < 0.005.

**P < 0.001.

[a]Each treatment tested in 30, 3-minute trials.

[b]The column for no response is omitted.

[c]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Table 4-6. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected synthetic monoolefins.

Treatment[a]	Amount	No. Responding	Frequency of Response[b]			Response[c] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	29**	10	15	4	52
Hexane-treated T male (control)	2 µl	3	3	0	0	3
(Z)-9-tricosene	40 µg	3	3	0	0	3
(Z)-5-tricosene	40 µg	8	8	0	0	8
(Z)-9-pentacosene	40 µg	17**	17	0	0	17
(Z)-9-heptacosene	40 µg	12*	11	1	0	13

*P < 0.01.

**P < 0.001.

[a]Each treatment tested in 30, 3-minute trials.

[b]The column for no response is omitted.

[c]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

to (Z)-9-tricosene was not different from the response to T male controls while (Z)-5-tricosene received a slight but not statistically significant male response (x^2 = 2.78, N.S.). In each case the hierarchy of response by males to these monoolefins was statistically different from the hierarchy of response to live females; none of the monoolefins tested singly caused substantial (++) or (+++) behavior.

The number of males responding to (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene in combination (Table 4-7) was significantly greater than the number of males responding not only to controls but also to each of the monoolefins tested singly [monoolefin combination versus (Z)-5-tricosene, x^2 = 19.46, $P < 0.001$; versus (Z)-9-pentacosene, x^2 = 7.50, $P < 0.01$; versus (Z)-9-heptacosene, x^2 = 4.02, $P < 0.05$]. However, the hierarchy of male response to this combination of monoolefins was statistically different from the response to live females in the same bioassay series (x^2 = 16.70, $P < 0.001$).

The number of males responding to combinations of two of these monoolefins was in each case significantly greater than the response to hexane-treated T males (Table 4-8). The combination (1:1:1) of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene at 40 µg induced the most responding males of any treatment tested during these bioassays. Also, the response score for this combination was 48 versus a score of 55 for live females. While the hierarchy of response for each of the combinations of 2 monoolefins in this bioassay series was statistically different than the live female, the hierarchy of response to (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene in combination was not statistically different from the response hierarchy seen for the live female (x^2 = 5.02, N.S.).

Table 4-7. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected synthetic monoolefins.

Treatment[a]	Amount	No. ♂♂ Responding	Frequency of Response[b]			Response[c] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	30***	10	18	2	50
Hexane-treated T male (control)	2 µl	6	6	0	0	6
(Z)-5-tricosene	40 µg	8	8	0	0	8
(Z)-9-pentacosene	40 µg	15*	14	1	0	16
(Z)-9-heptacosene	40 µg	18**	17	1	0	19
(Z)-5-tricosene (Z)-9-pentacosene (Z)-9-heptacosene (1:1:1 mixture)	45 µg	25***	19	4	2	33

*P < 0.025.

**P < 0.005.

***P < 0.001.

[a]Each treatment tested in 30, 3-minute trials.

[b]The column for no response is omitted.

[c]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Table 4-8. Hierarchy of courtship behavior of adult male H. irritans to T males treated with selected synthetic monoolefins.

Treatment[a]	Amount	No. Responding	Frequency of Response[b]			Response[c] Score (max. = 90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Live female (control)	--	29*	7	18	4	55
Hexane-treated T male (control)	2 µl	3	3	0	0	3
(Z)-5-tricosene (Z)-9-pentacosene (1:1 mixture)	40 µg	15*	10	4	1	20
(Z)-5-tricosene (Z)-9-heptacosene (1:1 mixture)	40 µg	21*	14	6	1	28
(Z)-9-pentacosene (Z)-9-heptacosene (1:1 mixture)	40 µg	20*	15	3	2	25
(Z)-5-tricosene (Z)-9-pentacosene (Z)-9-heptacosene (1:1:1 mixture)	40 µg	26*	10	10	6	48

*$P < 0.001$.

[a]Each treatment tested in 30, 3-minute trials.

[b]The column for no response is omitted.

[c]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Discussion

Development of this bioassay permitted testing of horn fly cuticular lipids for mating stimulant activity. The effective lower limit for the dose of synthetic test compounds on T males in this bioassay was between 10 μg and approximately 40 μg corresponding to about 1-4 fly equivalents relative to female total hydrocarbon. Lesser quantities of test compounds did not induce consistent male response (Table 4-5).

The observed stimulation of male courtship behavior by the natural paraffin and monoolefin fractions of the female cuticular hydrocarbons or combinations of these fractions agrees with reports on mating stimulant pheromones of other species in the family Muscidae. At the doses tested, the natural paraffin and monoolefin fractions were biologically active in inducing male courtship behavior when bioassayed alone or in combination. The monoolefins (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene are horn fly cuticular monoolefins responsible for releasing male courtship behavior. Because the number of responding males was greatest to this combination of compounds and the hierarchy of male response to this combination most closely resembled that observed for live females, the mating stimulant pheromone of the horn fly appears to be a complex of compounds, perhaps implicating other olefins, or other compounds such as those found in small quantities in house flies including methyl-branched paraffins or long chain hydrocarbons with hetero-atoms such as epoxides, ketones, or esters (Uebel et al., 1978c).

As all olefins described here are present in both sexes in different quantities a more critical assignment of the role of each compound has yet to be defined. We view this chemically induced pheromone

activity of the horn fly as mating stimulation as this term describes the behavior of the male seen in our bioassay apparently only due to the presence of these chemicals.

Investigations of the sex pheromones of other Muscids including the house fly (Uebel et al., 1976; Uebel et al., 1978c), the face fly (Uebel et al., 1975a; Sonnet et al., 1975), and the stable fly (Uebel et al., 1975b; Sonnet et al., 1977; Sonnet et al., 1979) have established that sex pheromones of the Muscids, in these three species at least, are more complex than previously envisioned; branched paraffins in the house fly and stable fly appear to act as synergists of olefins, and,in particular, act primarily as mating stimulants which operate at close range; non-hydrocarbons in the house fly may play a mating stimulant role; and several monoolefins are each active as mating stimulants in the face fly and stable fly. The precise role that individual compounds or combinations play in the progressive hierarchy of response that occurs during courtship has yet to be defined.

CHAPTER 5

THE MATING STIMULANT PHEROMONE OF THE HORN FLY, *Haematobia irritans* (L.): THE EFFECT OF VARYING CONCENTRATION ON THE HIERARCHY OF COURTSHIP BEHAVIOR

Abstract

Florida Laboratory Strain (F) males and females and Florida Wild Strain (W) (field-collected) males and females had similar analytical gas chromatograms for total paraffins. The F and W females had relatively greater quantities of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene than did F and W males; F and W males had relatively greater quantities of (Z)-9-tricosene. Bioassays utilizing the response of virgin males to treated, virgin male horn flies previously washed with hexane indicated that (Z)-9-tricosene did not attenuate the courtship behavior of males toward dead females. Additionally, these bioassays demonstrated that with increasing doses of a 1:1:1 combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene, the frequency of successive elements of the courtship hierarchy of the male was increased.

Introduction

Sex pheromones for a number of species in the family Muscidae have been identified as has been detailed in Chapters 2 and 4. The bioassays

described in Chapter 4, which utilized the response of virgin male horn flies toward other virgin male horn flies treated with female paraffins and selected monoolefins, indicated that the combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene (1:1:1) was responsible for releasing male courtship behavior at doses between 10 μg and 40 μg.

Wood et al. (1970) hypothesized that different pheromone concentration thresholds were probably required for the initiation of various steps in the sexual behavior of many insect species. Shorey (1973) reviewed research studies in which increasing pheromone concentrations were demonstrated to influence the initiation of successive elements in hierarchy of sexual behavior. In Lepidopteran species, only low pheromone concentrations were necessary to initiate the flight of the male located at some distance from the female; however, higher pheromone concentrations were needed to induce male copulatory behavior once the male had approached the female.

In the normal behavior of a species, close-range stimuli may play an important role in the hierarchy of courtship behavior. But in the laboratory, female sex pheromones at the proper concentration have been demonstrated to release male copulatory behavior in the absence of other female-produced stimuli (Shorey, 1973; Young and Silverstein, 1975).

Bartell and Shorey (1969) demonstrated that successive steps in the mating sequence of the light-brown apple-moth, Epiphyas postivittana, were elicited when males were exposed to increasing concentrations of the female sex pheromone. This study also underscored the difficulties associated with interpreting features of the dose response data obtained

in this type of study. Carlson et al. (1978) demonstrated a dose response relationship in the hierarchy of the mating response of male Glossina morsitans morstians Westwood to increasing concentrations of one component of the female sex pheromone.

The objectives of this study were to quantitate the amount of major paraffins and monoolefins in horn flies of the Florida Laboratory Strain (F) and Florida Wild Strain (W) (field-collected) horn flies, to conduct bioassays to further define the involvement of selected cuticular hydrocarbons in the courtship behavior of the horn fly, and to investigate the effect of varying concentrations of a combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on the hierarchy of courtship behavior of the horn fly.

Materials and Methods

Horn flies used in these studies were from the laboratory colony maintained at the University of Florida (Florida Laboratory Strain, F) as described by Greer (1975) or from collections of wild horn flies (Florida Wild Strain W) from herds of cross-bred Angus cows in Alachua, Co., Florida. All flies were handled by the procedures described in Chapters 3 and 4. Florida Laboratory Strain (F) adults were used in bioassays and were a source of crude lipid extracts. Florida Wild Strain (W) adults were used only as a source of crude lipid extracts.

Crude lipid extracts were obtained from 3-7 day old males and females of the F strain; the age of the field-collected W strain flies was undetermined. Field-collected flies were sexed in the laboratory

before being extracted. Flies to be extracted were lightly anesthetized with carbon dioxide and rinsed with a volume of hexane equivalent to 4 ml per fly. After the extract was filtered through a Whatman No. 4 filter, the hexane was removed under vacuum in a rotary evaporator and by evaporation with a stream of nitrogen.

Lipid extracts were fractionated by liquid chromatography on 1 cm ID x 45 cm columns of activated silica gel (60-200 mesh, J.T. Baker Chemical Co., Phillipsburg, NJ). The hydrocarbon fraction was eluted with 50 ml of hexane. More polar fractions were eluted with additional 50 ml volumes of hexane with increasing quantities of ether (5%, 10%, 50%). Separation of hydrocarbons from other lipid classes was confirmed by thin layer chromatography (TLC) on activated silica gel plates (250 μ Anasil, Analabs, Inc., North Haven, CT) developed with a hexane:ether:acetic acid (90:10:1 v/v) solvent mixture.

The hydrocarbon fraction was separated into fractions by liquid chromatography on 1 cm ID x 45 cm columns packed with 20% silver nitrate impregnated silica gel (60-200 mesh, Hi-Flosil-Ag, Applied Science Laboratories Inc., State College, PA). The paraffins were eluted in the first fraction with 50 ml of hexane; the monoolefins were eluted in the fourthfraction with a 50 ml volume of 5% ether in hexane. Separation of fractions was confirmed by TLC on silica gel plates impregnated with 20% silver nitrate (250 μ Uniplates, Analtech Inc., Newark, DE) developed with a hexane:benzene (75:25 v/v) solvent mixture. All hexane used in these investigations was stirred over concentrated sulfuric acid for 2 days, washed with distilled water, distilled over sodium, and stored over sodium hydroxide.

The hydrocarbon fractions were quantitated by analytical gas chromatography (Varian Model 2100 and Varian Aerograph Series 1200 with glass columns, 1.8 m x 2 mm ID containing 3% OV-1 on 100-120 mesh Gas Grhom Q, and flame ionization detector) so that comparisons could be made to the quantifications made by Mackley (1977). The chain lengths of paraffins and monoolefins were determined by injection of known paraffin standards so that Kovats' indices (1965) could be assigned. Quantifications of compounds were made by comparing peak areas with the peak areas of known concentrations of standards on a Hewlett-Packard Model 3380A Integrator.

Two types of bioassays were conducted during these studies. In the first type, lipid hydrocarbon fractions or synthetic monoolefins, whose identification and synthesis have been described by Mackley (1977), were bioassayed as described in Chapter 4; T males, however, were prepared by rinsing these flies with a volume of hexane equivalent to 4 ml per fly, to remove male lipids from the T male prior to applying the candidate compounds for bioassay. Otherwise, the procedures, experimental conditions, and analyses of data were as previously described.

In the second type of bioassay, the relationship between varying the concentration of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene and the frequency of the elements in the hierarchy of courtship behavior was investigated. In general, the procedures for this dose-response bioassay were the same as those for the first bioassay type with the following exceptions: Varying doses of the 1:1:1 combination of monoolefins prepared by serial dilution were applied by syringe to the dorsum of hexane-rinsed T males.

The treated T male was positioned on the bioassay apparatus described in Chapter 4. Five 3-8 day old virgin males were transferred into a screened-topped, clear plastic specimen container through a circular hole in the bottom of the container. The response to each dose was evaluated by counting the frequencies of the following courtship elements that were displayed by the 5 males toward the treated T male: 1) strike (+), an attempt by the virgin male to get onto the dorsum of the T male; 2) arrested movement (++), after striking the T male, the responding male maintained contact with the T male; 3) positioning on the dorsum (+++), after striking the T male, the responding male made characteristic grasping movements with its legs around the T male and positioned its genitalia below the terminal portion of the T male's abdomen. The number of copulations which occurred was also recorded.

A 10-minute bioassay period began after the specimen container holding the virgin males was placed over the T male. For each dose, fifteen10-minute trials were conducted. The order of testing the doses within each trial was randomly determined. Similar trials were also conducted on hexane-rinsed males and dead females prepared by brief exposure to -15°C.

Before these data were analyzed, they were converted to the number of courtship elements per hour. Linear and quadratic regression analyses (Helwig and Council, 1979) were conducted for the frequency of each element of the courtship hierarchy versus the dose of the monoolefin mixture applied to the T male. Probit analyses were also conducted on the same data. For each element of the courtship hierarchy, the percentage of the trials in which each element was observed was analyzed as a function of the dose applied to the T male.

Results

Analytical gas chromatograms of the total paraffins of the F males, F females, W males, and W females are illustrated in Figures 5-1, 5-2, 5-3, and 5-4, respectively. Quantifications of the total paraffins and major individual components for each group of flies listed above are presented in Tables 5-1 and 5-2, respectively.

The total paraffins comprised 31.6%, 3.07 µg per fly; 21.4%, 2.78 µg per fly; 58.7%, 2.29 µg per fly; and 38.8%, 2.90 µg per fly, of the total hydrocarbons of the F males, F females, W males, and W females, respectively (Table 5-1). The majority of components were odd-numbered, straight-chain paraffins 21 to 31 carbons in length. Smaller quantities of even-numbered straight-chain paraffins 22 to 28 carbons in length and methyl-branched paraffins were present. Qualitatively, the chromatographic profiles of the paraffins were similar for all groups of flies; however, quantitative differences did exist (Table 5-2). In the F males the C_{21} and C_{23} straight chain paraffins were more abundant than in the F females in which the C_{25}, C_{27}, and C_{29} straight-chain paraffins were relatively more abundant. The W males and W females had similar quantities of the straight-chain paraffins and more closely resembled the F females than the F males.

Analytical gas chromatograms of the total monoolefins of the F males, F females, W males, and W females are illustrated in Figures 5-5, 5-6, 5-7, and 5-8, respectively. Quantifications of the total monoolefins and major individual components for each source of flies listed above are presented in Tables 5-1 and 5-3, respectively.

The total monoolefins comprised 68.4%, 6.62 µg per fly; 78.6%, 10.21 µg per fly; 41.3%, 1.61 µg per fly; and 61.2%, 4.57 µg per fly, of the total hydrocarbons of the F males, F females, W males, and W

Figure 5-1. Analytical gas chromatogram of total paraffins recovered from cuticular rinses of virgin male 6-7 day old F. horn flies: 1.0 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

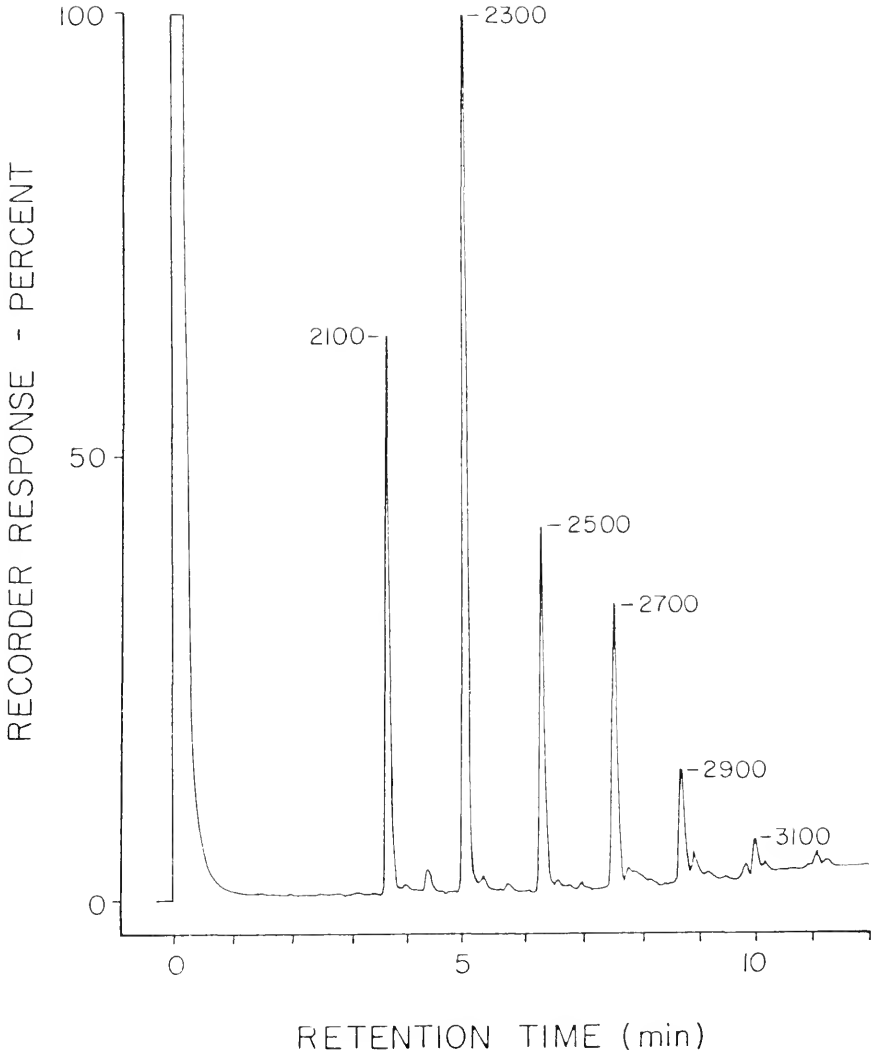

100
50
0
RECORDER RESPONSE - PERCENT
2100
2300
2500
2700
2900
3100
0
5
10
RETENTION TIME (min)

Figure 5-2. Analytical gas chromatogram of total paraffins recovered from cuticular rinses of virgin female 6-7 day old F. horn flies: 0.8 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

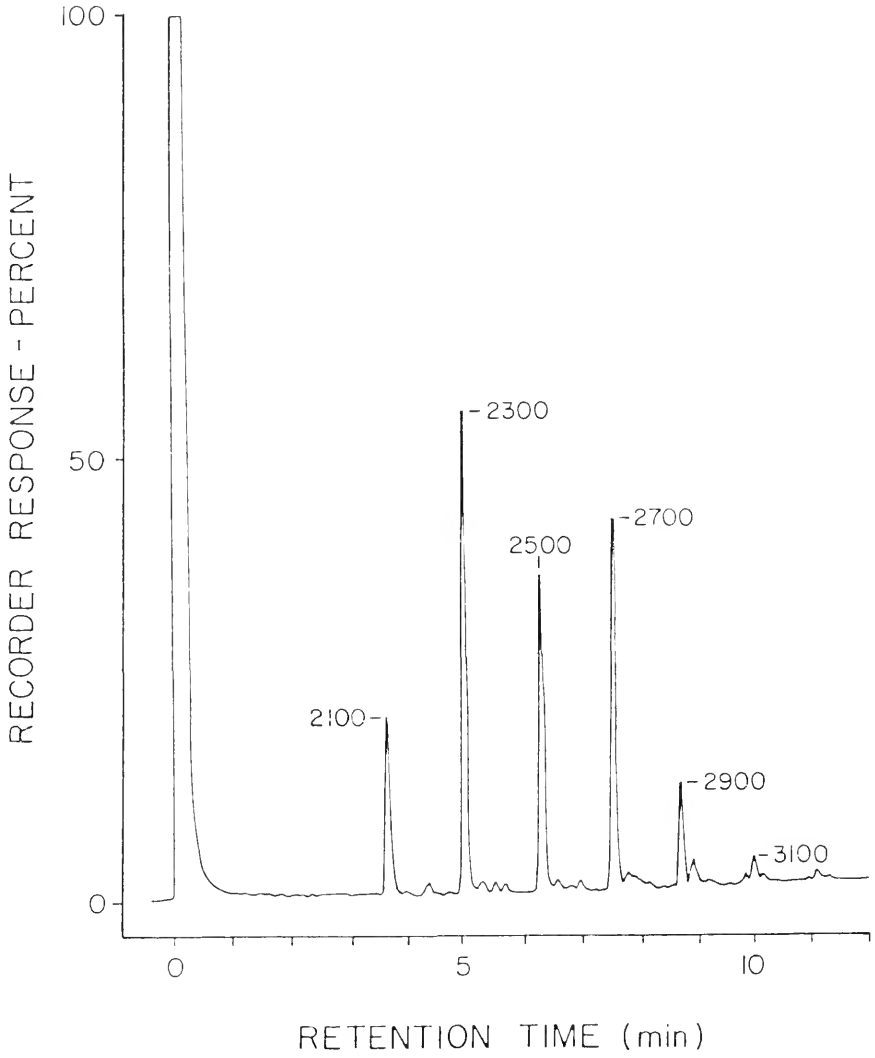

100
50
0
RECORDER RESPONSE - PERCENT
2100
2300
2500
2700
2900
3100
0
5
10
RETENTION TIME (min)

Figure 5-3. Analytical gas chromatogram of total paraffins recovered from cuticular rinses of field-collected male *H. horn* flies: 1.0 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10-11; injection port temperature 260°C; and detector temperature 320°C.

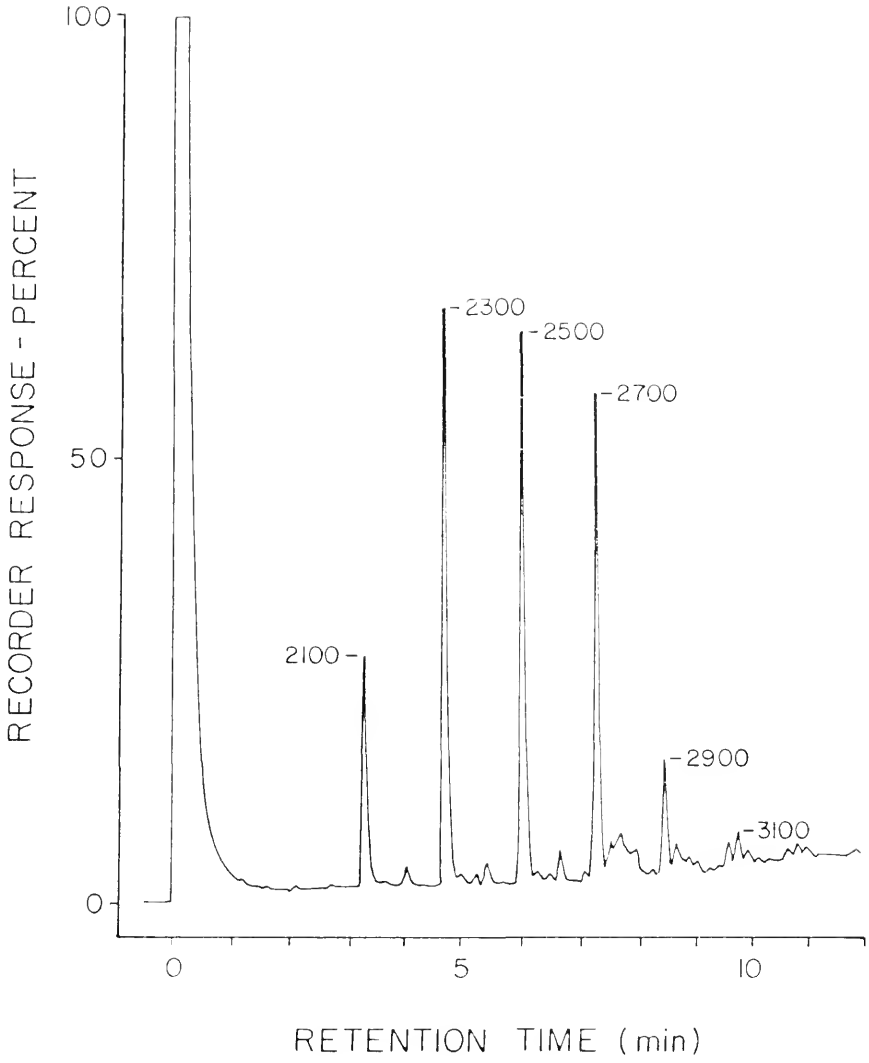

100
50
0
RECORDER RESPONSE - PERCENT
2100
2300
2500
2700
2900
3100
0
5
10
RETENTION TIME (min)

Figure 5-4. Analytical gas chromatogram of total paraffins recovered from cuticular rinses of field-collected female W horn flies: 0.9 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12 per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

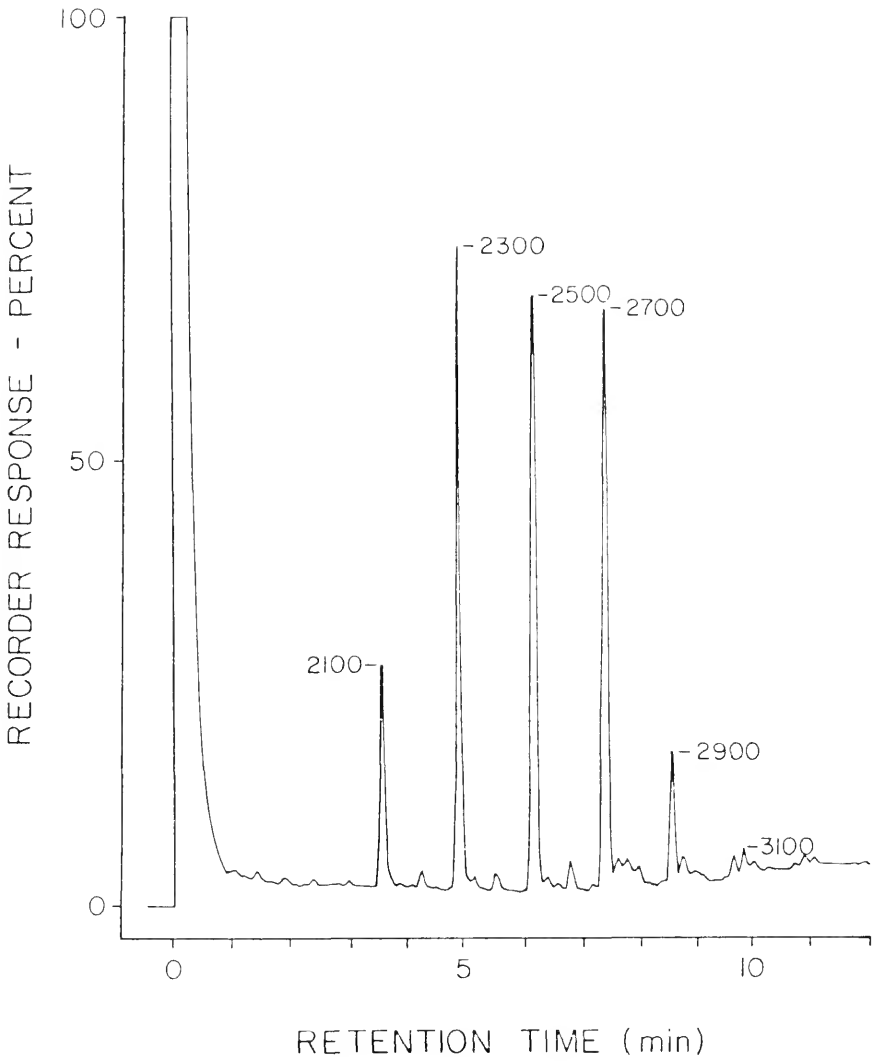

100
50
0
RECORDER RESPONSE - PERCENT
2100
2300
2500
2700
2900
3100
0
5
10
RETENTION TIME (min)

Table 5-1. Quantification of total paraffins and monoolefins recovered from hexane cuticular rinses of adult horn flies.

Source	Age (days)	µg of Component Recovered per Fly		
		Paraffins	Monoolefins	Total Hydrocarbons
F Males	6-7	3.07	6.62	9.69
F Females	6-7	2.78	10.21	12.98
W Males (field-collected)	unknown	2.29	1.61	3.90
W Females (field-collected)	unknown	2.90	4.57	7.47

Table 5-2. Quantification[a] of major paraffins recovered from hexane cuticular rinses of adult horn flies.

Kovats' Index	Source			
	Florida Laboratory Strain (F)		Florida Wild Strain (W)	
	Male	Female	Male	Female
2100	0.56	0.23	0.27	0.23
2130	0.00	0.00	0.01	0.01
2200	0.03	0.02	0.03	0.02
2300	1.00	0.63	0.69	0.68
2330	0.02	0.02	0.02	0.02
2400	0.01	0.01	0.03	0.02
2500	0.38	0.42	0.64	0.63
2530	0.02	0.02	0.02	0.02
2600	0.01	0.02	0.04	0.03
2700	0.30	0.49	0.57	0.61
2730	0.02	0.02	0.05	0.04
2750	0.04	0.03	0.11	0.06
2800	0.00	0.01	0.06	0.02
2900	0.12	0.14	0.14	0.14
2930	0.05	0.05	0.05	0.04
3100	0.04	0.04	0.04	0.03
3130	0.02	0.01	0.02	0.02

[a] µg

Figure 5-5. Analytical gas chromatogram of total monoolefins recovered from cuticular rinses of virgin male 6-7 day old *F.* horn flies: 0.5 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

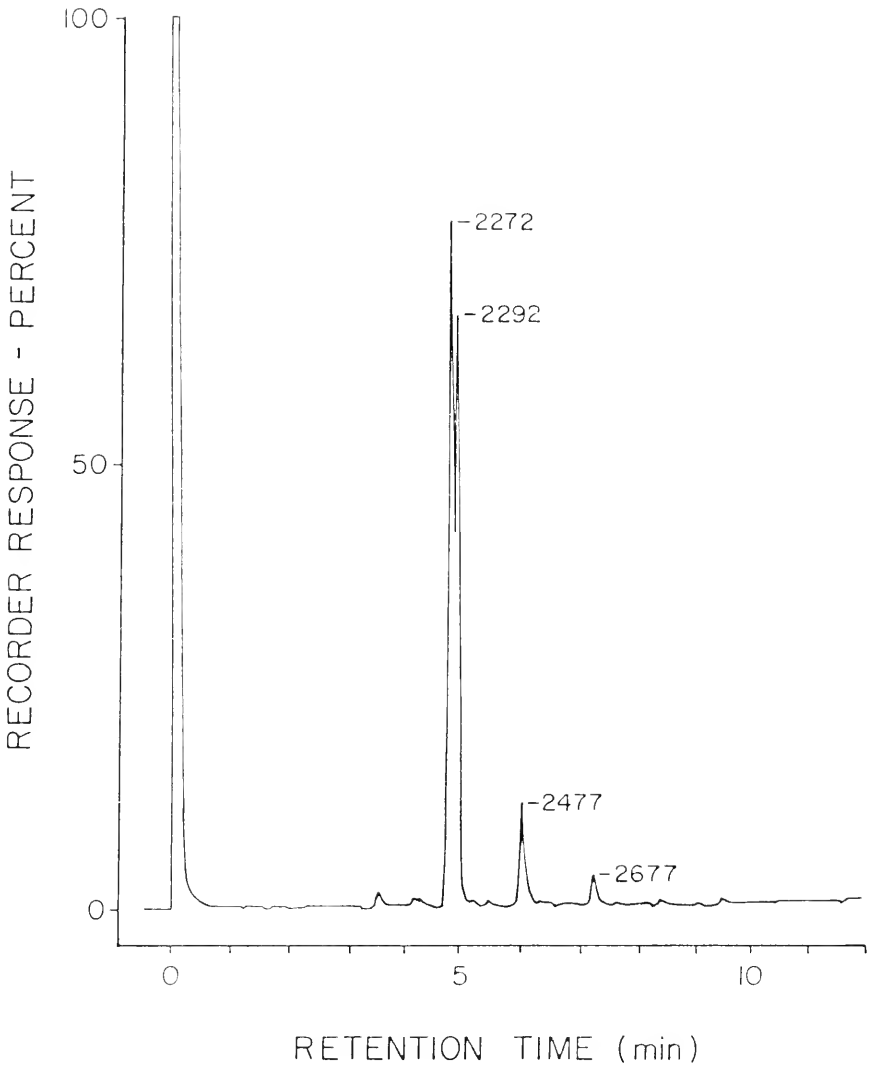

100
50
0
RECORDER RESPONSE - PERCENT
2272
2292
2477
2677
0
5
10
RETENTION TIME (min)

Figure 5-6. Analytical gas chromatogram of total monoolefins recovered from cuticular rinses of virgin female 6-7 day old $\bar{F}$ horn flies: 0.5 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

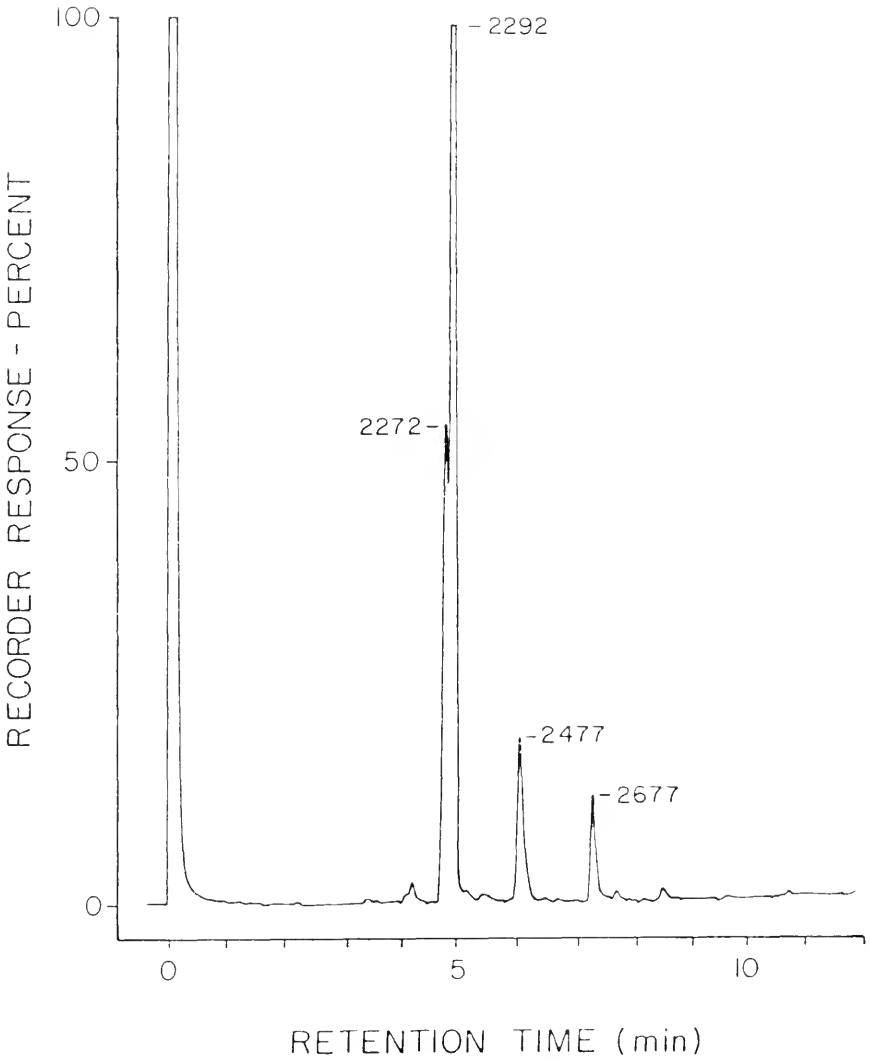

100
50
0
RECORDER RESPONSE - PERCENT
2292
2272
2477
2677
0
5
10
RETENTION TIME (min)

Figure 5-7. Analytical gas chromatogram of total monoolefins recovered from cuticular rinses of field-collected male *H. irritans* horn flies: 1.0 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

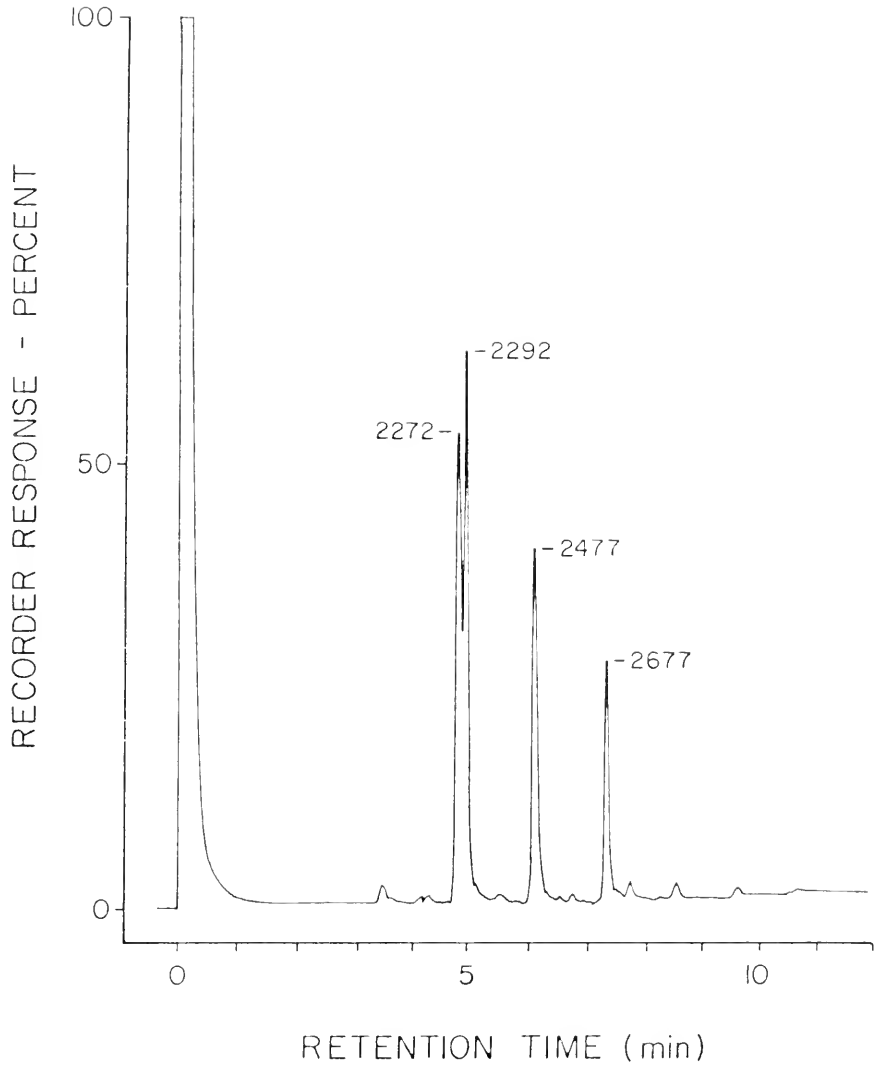

100
50
0
RECORDER RESPONSE - PERCENT
2272
2292
2477
2677
0
5
10
RETENTION TIME (min)

Figure 5-8. Analytical gas chromatogram of total monoolefins recovered from cuticular rinses of field-collected female W horn flies: 1.0 µl sample rinse in hexane injected on a glass column, 1.8 m x 2 mm ID, filled with 3% OV-1 on Gas Chrom Q, 100-120 mesh. Chromatogram was temperature programmed from 200-325°C at 12° per min; detector attenuation 1 x 10^{-11}; injection port temperature 260°C; and detector temperature 320°C.

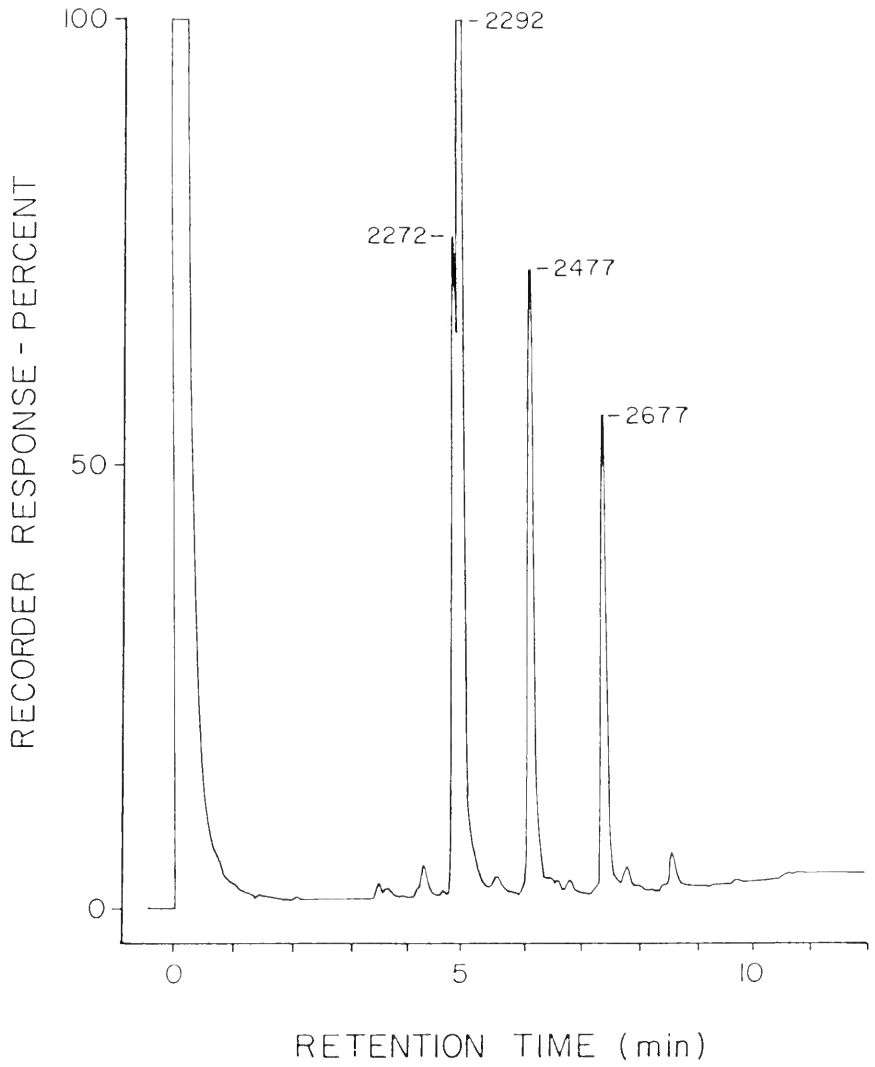

100
50
0
RECORDER RESPONSE - PERCENT
2272
2292
2477
2677
0
5
10
RETENTION TIME (min)

Table 5-3. Quantification of the major monoolefins recovered from hexane cuticular rinses of adult horn flies; 6-7 day old male and female F strain and W (field-collected) horn flies.

Kovats' Index	Compound	Florida Laboratory Strain (F)				Florida Wild Strain (W)			
		µg of Component Recovered per Fly		Percent of Total Monoolefins Recovered		µg of Component Recovered per Fly		Percent of Total Monoolefins Recovered	
		Male	Female	Male	Female	Male	Female	Male	Female
2272	(Z)-9-tricosene	3.20	2.26	48.4	22.1	0.39	0.58	24.0	12.6
2292	(Z)-5-tricosene	2.32	6.00	35.1	58.8	0.52	2.34	32.1	51.1
2477	(Z)-9-pentacosene	0.56	0.90	8.4	8.8	0.35	0.81	21.8	17.8
2677	(Z)-9-heptacosene	0.16	0.52	2.4	5.1	0.23	0.56	14.6	12.3
	Totals	6.24	9.68	94.3	94.8	1.49	4.29	92.5	93.8

females, respectively (Table 5-1). In each group of flies, 4 major compounds were present with Kovats' indices of 2272, 2292, 2477, and 2677. These compounds comprised 94.3%, 94.8%, 92.5%, and 93.8% of the F males', F females', W males', and W females' total monoolefin fraction (Table 5-3), respectively. Mackley (1977) had previously identified these 4 compounds as (Z)-9-tricosene (2272), (Z)-5-tricosene, (2292), (Z)-9-pentacosene (2477), and (Z)-9-heptacosene (2677). The quantity of these 4 compounds varied with the source of the flies. In the F males and F females they accounted for 6.24 µg/fly and 9.68 µg/fly, respectively, while in W males and W females they accounted only for 1.49 µg/fly and 4.29 µg/fly, respectively. Both sources of females, however, had relatively more (Z)-5-tricosene than did their respective male source; in addition, both female sources had relatively more (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene than did males. Both sources of males had about 2x more (Z)-9-tricosene than did the corresponding females from the same source.

Previously conducted bioassays (Chapter 4) had indicated that the male crude lipid extract was not biologically active. When the male paraffin and monoolefin fractions were bioassayed individually (Table 5-4), no activity was found in either fraction. The number of males responding to the paraffin fraction (X^2 = 2.78, N.S.) and the monoolefin fraction (X^2 = 1.92, N.S.) were not significantly different than the number of males responding to the hexane-treated T male control.

Previously conducted bioassays (Chapter 4) had also indicated that at doses of 4 fly equivalents per unwashed T male, the paraffin fraction, the monoolefin fraction, and a 1:1 paraffin/monoolefin

Table 5-4. Hierarchy of courtship behavior of adult male H. irritans to hexane-washed T males treated with selected lipid fractions.[a]

Treatment[b]	Amount	No. ♂♂ Responding	Frequency of Response[e]			Response[f] Score (max.=90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Dead female[c] (control)	--	28	5	11	12	52
Hexane-washed T male (control)	2 µl (hexane)	3	3	0	0	3
Male hydrocarbon						
Paraffin fraction	4 F.E.[d]	8	8	0	0	8
Monoolefin fraction	4 F.E.	7	5	2	0	9

[a]Lipid fractions were obtained from 3-4 day old males of the Florida Laboratory Strain (F).

[b]Each treatment tested in 30 3-minute trials.

[c]Killed by freezing at -15°C and thawed before being bioassayed.

[d]F.E., fly equivalents.

[e]The column for no response is omitted.

[f]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

combination each gave a significantly greater number of male responses than did hexane-treated T males, but the paraffin/monoolefin combination did not produce a greater number of responding males than did the monoolefin fraction alone. A second series of bioassays was conducted to investigate the activity of the paraffin/monoolefin combination on hexane-washed T males. Because the majority of paraffins in F and W females were straight-chain (unbranched or normal) paraffins, a 1:3:2:2:1 combination of C_{21}, C_{23}, C_{25}, C_{27}, and C_{29} paraffin standards was prepared to bioassay. This ratio of paraffins approximately represented the ratio of the straight-chain paraffins found in F females and W females (Table 5-2). The bioassays (Table 5-5) indicated that significantly more males responded to the monoolefin combination ($X^2 = 15.86$, $P < 0.005$) and the 1:1 monoolefin/paraffin combination ($X^2 = 25.86$, $P < 0.005$) than to hexane-washed T males; however, when the paraffin combination was bioassayed on hexane-washed T males and on unwashed T males, the activity of the paraffin combination varied from bioassay to bioassay. The number of males responding to the paraffin combination applied to the unwashed T males ($X^2 = 14.28$, $P < 0.005$) was significantly greater than to hexane-washed T male controls. The number of males responding to the paraffin combination applied to hexane-washed T males ($X^2 = 6.65$, N.S.) was not significantly different than the number responding to hexane-washed T male controls. The number of males responding to the treatments of a dead female, a hexane-treated dead female, and a (Z)-9-tricosene treated dead female ($X^2 = 2.10$, N.S.) were not significantly different (Table 5-6.

Table 5-5. Hierarchy of courtship behavior of adult male H. irritans to hexane-washed T males treated with selected monoolefins and unbranched (normal) paraffins.

Treatment[a]	Amount	No. ♂♂ Responding	Frequency of Response[c]			Response[d] Score (Max.=90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Dead female[b] (control)	--	28*	5	19	4	55
Hexane-washed T male (control)	2 µl (hexane)	4	4	0	0	4
(Z)-5-tricosene (Z)-9-pentacosene (Z)-9-heptacosene (1:1:1 mixture)	40 µg	19*	13	5	1	26
(Z)-5-tricosene (Z)-9-pentacosene (Z)-9-heptacosene + Unbranched paraffins (C_{21}, C_{23}, C_{25}, C_{27}, C_{29}) (1:3:2:2:1 mixture)	20 µg + 20 µg	19*	18	1	0	19
Unbranched paraffins on unwashed T male (C_{21}, C_{23}, C_{25}, C_{27}, C_{29}) (1:3:2:2:1 mixture)	40 µg	13*	11	1	1	13

Table 5-5. continued

Treatment[a]	Amount	No. ♂♂ Responding	Frequency of Response[c]			Response[d] Score (Max.=90)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Unbranched paraffins on hexane-washed T male (C_{21}, C_{23}, C_{25}, C_{27}, C_{29}) (1:3:2:2:1 mixture)	40 µg	4	4	0	0	4

*$P < 0.005$.

[a]Each treatment tested in 30 3-minute trials.

[b]Killed by freezing at -15 C and thawed before being bioassayed.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

Table 5-6. Hierarchy of courtship behavior of adult male H. irritans to dead[a] female H. irritans treated with (Z)-9-tricosene.

Treatment[b]	Amount	No. Responding	Frequency of Response[c]			Response[d] Score (Max.=60)
			+ (strike)	++ (arrested movement)	+++ (positioning)	
Dead female[a]	--	18	2	15	1	35
Hexane-treated dead female	2 µl	20	2	17	1	39
(Z)-9-tricosene treated dead female	40 µg	20	2	14	4	42

[a]Killed by freezing at -15° C and thawed before being bioassayed.

[b]Each treatment tested in 20, 3-minute trials.

[c]The column for no response is omitted.

[d]The response score for each treatment was calculated by assigning 1 point for each (+), 2 points for each (++), and 3 points for each (+++).

The results of bioassays in which individual elements of the hierarchy of courtship behavior were measured when doses of a 1:1:1 combination (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene were applied to hexane-washed T males are presented in Figures 5-9, 5-10, and 5-11 and Table B-1. The responses of virgin males to dead female controls and hexane-washed male controls were determined to provide baseline measures of responsiveness of virgin males. Virgin males made an average of 1.4 strikes per hour on hexane-washed male controls but did not show any other courtship behavior toward hexane-washed males. However, virgin males made an average of 13.8 strikes, 10.6 arrested movements, 1.2 positionings on the dorsum, and 0.5 copulations per hour on dead female controls.

The relationship between the mean number of strikes per hour and the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on hexane-washed T males is illustrated in Figure 5-9. With increased doses of the monoolefin mixture the mean number of strikes per hour increased (Table B-1); a significant linear regression ($y = 9.46 + 6.75x$) adequately described the relationship. Figure 5-10 similarly illustrates the relationship between an increased dose of the monoolefin combination and the mean number of arrested movements per hour. A significant linear regression ($y = 1.51 + 1.90x$) adequately described the observed relationship. Although there is variability in the data from dose to dose, a greater number of strikes per hour were observed at the higher doses tested. Figure 5-11 illustrates the relationship between an increased dose of the monoolefin combination and the mean number of positionings on the dorsum. The actual number of responses per hour was low, but a greater

Figure 5-9. The relationship between the number of strikes per hour and the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on hexane-washed T males.

2.50
5.00
30.00
80.20

Figure 5-10. The relationship between the number of arrested movements per hour and the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on hexane-washed T males.

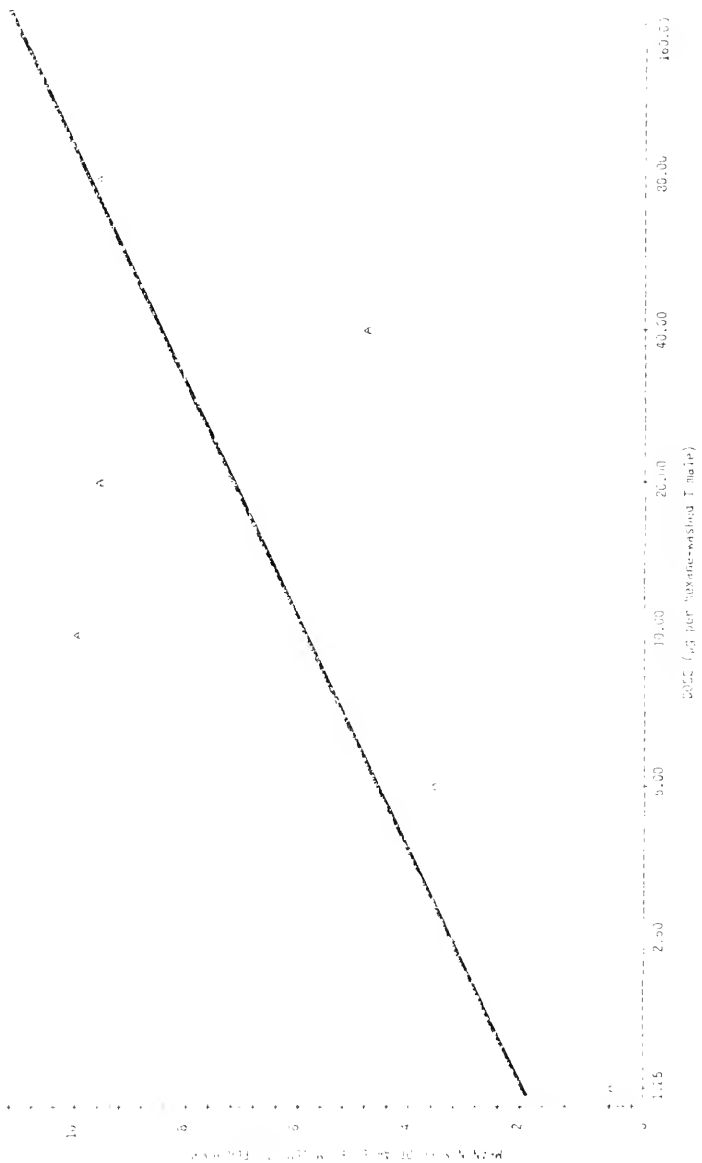
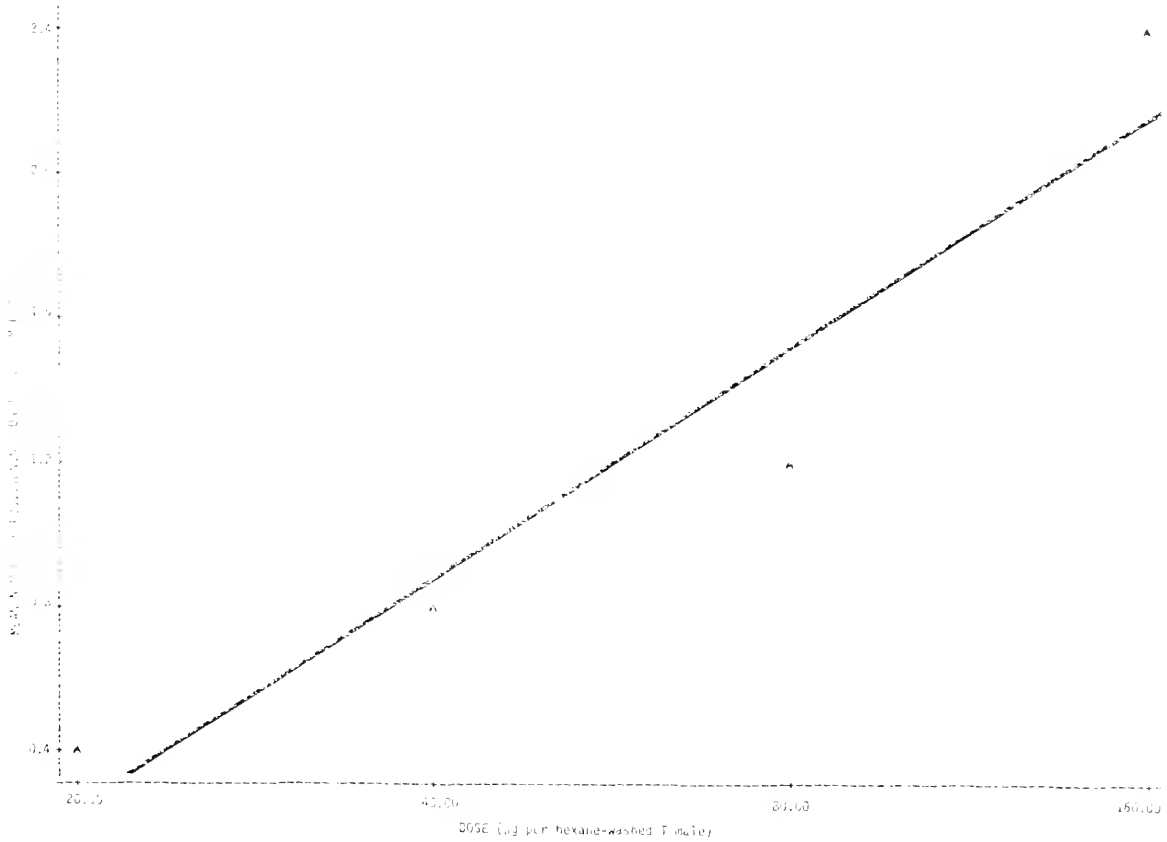

1.45
2.90
8.00
19.00
20.00
40.00
80.00
160.00
DOSE (g per 'standard' washing machine)

Figure 5-11. The relationship between the number of positionings on the dorsum and the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on hexane-washed T males.


2.4
0.4
40.00
80.00
160.00
DOSE (ug per hexane-washed T mole)

number of responses were seen as the dose was increased. In addition, positionings on the dorsum were only observed when the 4 highest doses were tested. A significant linear regression ($y = -2.53 + 0.92x$) adequately described the observed relationship.

The probit analyses conducted on the data obtained from the preceeding bioassays are illustrated in Figures 5-12, 5-13, and 5-14. For each dose of the monoolefin combination tested, the number of trials in which given elements of the courtship hierarchy occurred were tabulated (Table B-2). For each element of the courtship hierarchy, the percent of the trials in which that element occurred was analyzed versus the dose of the monoolefin combination applied to the hexane-washed T male. Parameters of these probit analyses are summarized in Table 5-7.

The number of trials in which strikes were observed increased with increasing doses of the monoolefin combination (Figure 5-12, Table B-2). At a dose of 20 µg per hexane-washed T male and higher doses, strikes were observed in all trials. The probit analyses for this relationship indicated a good fit of the data for normality. The probit analysis of the number of trials in which arrested movements were observed indicated a poor fit of the data for normality. As the data in Table B-2 and Figure 5-13 illustrate, the number of trials in which arrested movements were observed did not clearly increase with increasing doses of the monoolefin combination; however, the number of trials in which positionings on the dorsum were observed increased as the dose of monoolefin combination was increased (Figure 5-14, Table B-2). The probit analysis for these data indicated a good fit of the data for normality. The number of trials in which positionings on the

Figure 5-12. Probit analysis of the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene applied to hexane-washed T males versus the number of trials in which strikes were observed.

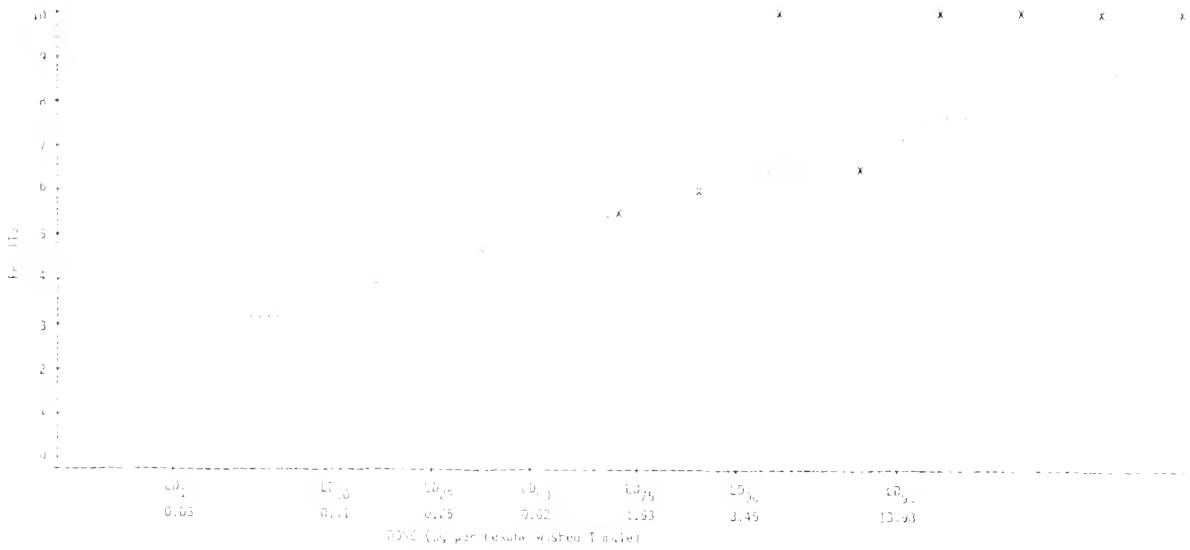
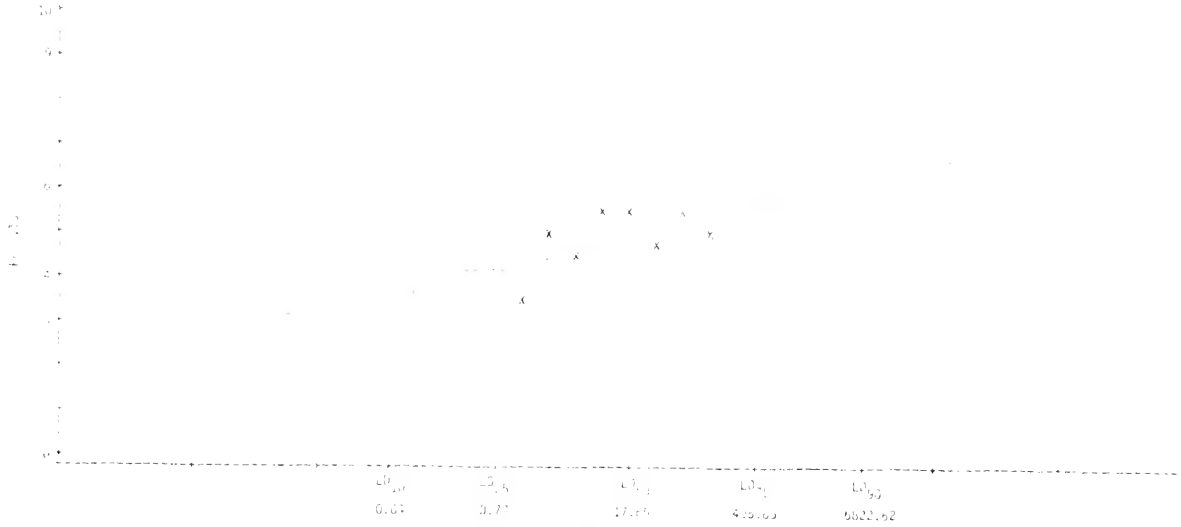

10
9
8
7
6
5
4
3
2
1
0
0.63
0.71
0.85
1.00
1.63
3.45
13.93

Figure 5-13. Probit analysis of the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene applied to hexane-washed T males versus the number of trials in which arrested movements were observed.



LD_{10}	LD_{50}	LD_{75}	LD_{95}	LD_{99}
0.03	0.77	17.58	439.03	5823.62

Figure 5-14. Probit analysis of the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene applied to hexane-washed T males versus the number of trials in which positionings on the dorsum were observed.

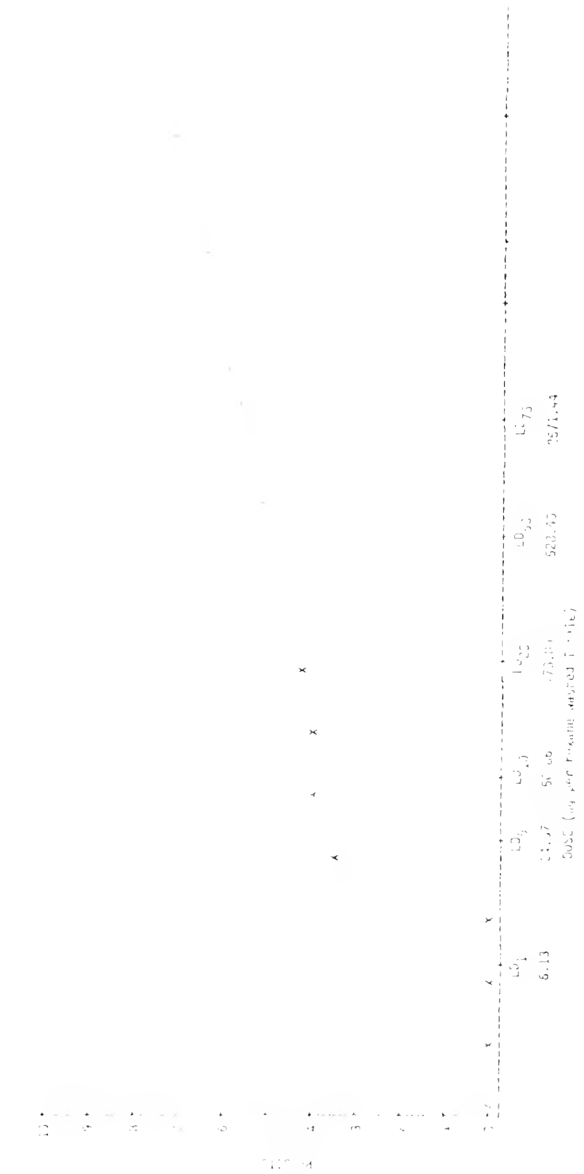

6.13

Table 5-7. Parameters of probit analyses of elements of the hierarchy of courtship response versus the dose of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene on hexane-washed T males.

		95 Percent Fiducial Limits	
Probability	Dose (μg)	Lower	Upper
Trials with Strikes			
0.01	0.03	0.00	0.19
0.10	0.11	0.00	0.44
0.25	0.25	0.00	0.73
0.50	0.62	0.02	1.31
0.75	1.53	0.31	2.71
0.90	3.45	3.13	43.94
0.99	13.98	6.13	809.99
Trials with Arrested Movements			
0.10	0.04	--	1.51
0.25	0.77	--	6.89
0.50	17.65	--	--
0.75	405.85	39.66	--
0.90	6822.62	172.94	--
Trials with Positionings on the Dorsum			
0.01	6.13	0.00	18.88
0.05	24.37	1.35	53.98
0.10	50.86	13.86	157.03
0.25	173.89	75.58	8402.90
0.50	528.43	195.38	1782202.19
0.75	2671.44	450.47	423833531.42

dorsum occurred was low and did not occur until the dose applied to the hexane-washed T male was 20 µg or greater.

Discussion

The analytical gas chromatograms and quantifications of the total hydrocarbons, paraffins, and monoolefins were similar to the findings of Mackley (1977). These studies, however, indicated a 1:3.67 ratio of total paraffins to total monoolefins in the F females in comparison to a 1:1.23 ratio found by Mackley (1977) for the same strain. Similarly, these studies demonstrated a 1:1.58 ratio of total paraffins to total monoolefins in the W females versus a 1:0.43 ratio found by Mackley (1977) for F_1 females of field-collected horn flies.

The quantifications of major paraffin components were similar for each group of flies studied. The F males did show greater quantities of straight-chain C_{21} and C_{23} paraffins than did F females, but W males and W females had similar quantities of most major paraffin components. Mackley (1977) had observed striking differences in the quantities of paraffin components between laboratory and field-collected horn flies and between the males and females of field-collected flies. These studies did not confirm the differences between similar sources and between the sexes as seen by Mackley (1977). These differences may be a result of the sources of horn flies for each study; the F_1 generation of field-collected horn flies was investigated by Mackley (1977) while field-collected horn flies that were of unknown age and that had been in contact with flies of the opposite sex were used in these studies.

In F and W flies, males tended to have relatively more (Z)-9-tricosene than did females while females had relatively more (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene. This finding is consistent with that of Mackley (1977); however, his field-collected females had virtually no (Z)-9-tricosene or (Z)-5-tricosene.

The unbranched paraffins which were bioassayed were not active in releasing male courtship behavior when applied to hexane-washed T males, but were active when applied to unwashed T males. The apparent activity of the unbranched paraffins on the unwashed T males may be due to the presence of the naturally occurring hydrocarbons on the T male. The application of 40 µg of (Z)-9-tricosene, a monoolefin found in greater relative amounts in males, to females did not attenuate the activity of the mating stimulant compounds naturally present on the female.

The 1:1:1 combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene was active in releasing male horn fly courtship behavior when applied to hexane-washed T males. Regression and probit analyses demonstrated that with increasing doses of the above described monoolefin combination the frequency of elements of the courtship hierarchy increased. Higher doses did release greater frequencies of successive elements in the hierarchy. At the highest dose tested, the courtship behavior released by the combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene did not equal that of the dead female controls. Other compounds may play a role in mating stimulation in the horn fly as previously suggested in Chapter 4. Other factors, such as close-range stimuli from the living female (Shorey, 1976), may play an important role in conjunction with the mating stimulant pheromone in releasing successive steps in the normal courtship hierarchy of the horn fly.

CHAPTER 6

EVALUATION OF THE MATING STIMULANT PHEROMONE OF THE HORN FLY, *Haematobia irritans* (L.), AS AN ATTRACTANT

Abstract

A combination of 3 cuticular monoolefins previously demonstrated to be active as a mating stimulant for male horn flies, *Haematobia irritans* (L.), was tested as an attractant to horn flies in laboratory olfactometer studies and in simulated field trials conducted in outdoor screened enclosures. The combination of (*Z*)-5-tricosene, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene was attractive to virgin male horn flies in olfactometer trials; however, in a simulated field trial, the combination was not attractive to either sex at the dose tested.

Introduction

Kinzer et al. (1970) demonstrated that 8-12 hour old male and female horn flies were attracted to cow and human odors in olfactometer studies. Using an olfactometer of a different design, Mackley (1977) determined that virgin 3-4 day old female horn flies were slightly attracted to female crude lipids, total horn fly hydrocarbons, and total horn fly monoolefins. Male horn flies were only attracted to (*Z*)-5-tricosene. The combination of the monoolefins (*Z*)-5-tricosene, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene has been demonstrated to be a mating stimulant for male horn flies (Chapter 4); however, Mackley did not test this combination in olfactometer trials.

The sex pheromones of several muscoid flies have been evaluated as attractants in field trials. Carlson et al. (1973) demonstrated that the addition of muscalure [(Z)-9-tricosene] to baits in several types of fly traps increased the catches of both males and females up to 12.4x more than catches in traps in which no muscalure was added to the baits. Previous olfactometer studies in the laboratory had demonstrated only the attraction of males (Carlson et al., 1974). Morgan et al. (1974) confirmed the effectiveness of muscalure as an attractant for both male and female house flies in further field trials; both sexes were caught in about equal numbers in traps which contained muscalure treated baits. In simulated field trials, Uebel et al. (1978) found that Fannia canicularis and F. pusio males, but not females, were slightly attracted to their respective mating stimulant pheromone; however, the mating stimulant pheromone of F. femoralis did not increase the trap catches of either males or females of that species.

This paper reports the results of the evaluation of the mating stimulant pheromone of the horn fly as an attractant in laboratory olfactometer studies and a simulated field trial.

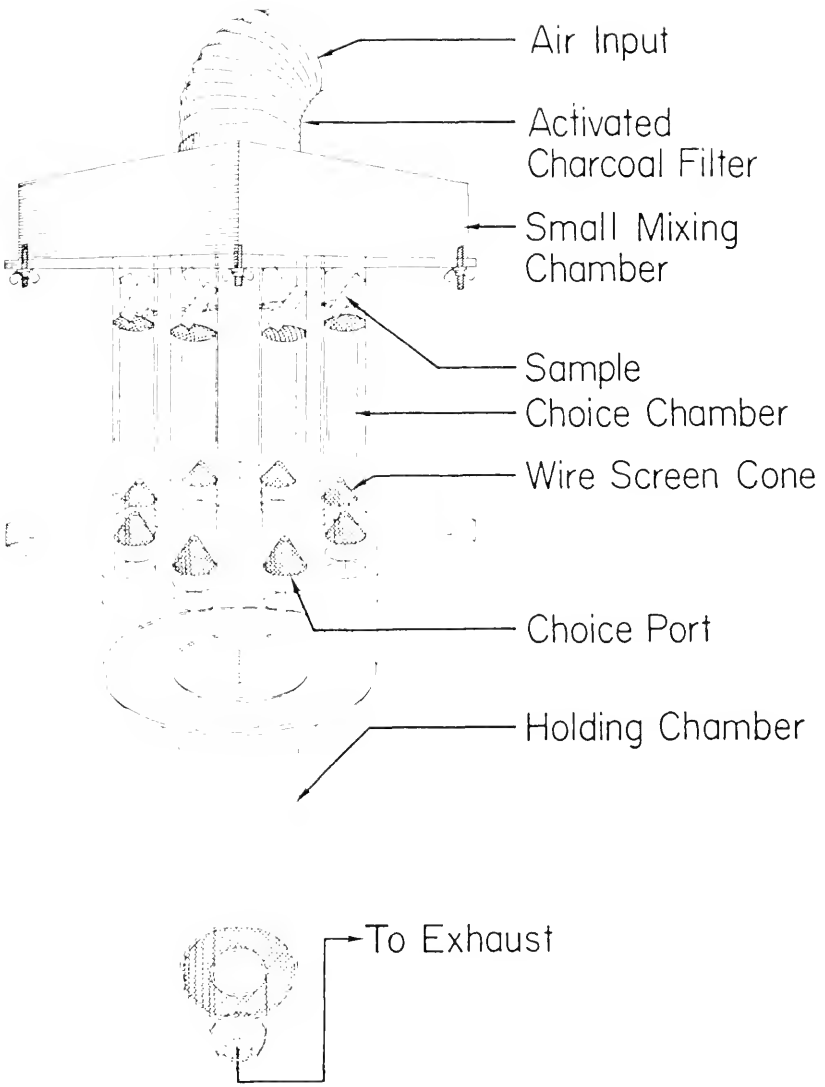
Materials and Methods

Horn flies used in these experiments were reared and handled as described by Greer (1975) and by the procedures described in Chapter 3. The combination of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene as a 1:1:1 mixture was tested in an olfactometer and a simulated field trial. These synthetic monoolefins were those whose identification and synthesis have been described by Mackley (1977).

The response of horn flies in the laboratory to test compounds was evaluated in a olfactometer (Figure 6-1) previously used and described by Mackley (1977). Modifications to the original design suggested by Mackley (1977) were made to improve the unit for use in these trials. The olfactometer consisted of a holding chamber (48 cm long, 11.5 cm ID) and 8 choice chambers (each 30 cm long, 3.5 cm ID). Insects moving vertically against an air flow from a plastic specimen container attached at the bottom of the holding chamber could pass through any of the 8 choice ports into the respective choice chamber above it. Insects entering a choice chamber passed through an 8 mm diameter hole at the apex of an inverted wire screen cone placed at each choice port. The cone and wire screen barrier placed near the top of each choice chamber kept responding insects within the choice chamber they had entered.

Partially heated and humidified air was directed past a heating element connected to a proportional temperature controller (YSI Model 73A, Yellow Springs Instrument, Yellow Springs, OH), through an activated charcoal filter and into a mixing chamber (21 x 21 x 9.5 cm) above the 8 choice chambers. Wet and dry bulb thermister probes were used to monitor the temperature and humidity at the mixing chamber; temperature and humidity were maintained at 27 ± 1°C and 90 ± 10%. The heated and humidified air passed into each of the 8 choice chambers over Whatman No. 4 filter papers (4.5 cm dia) previously treated with hexane or hexane solutions of the monoolefin mixture, through the holding chamber, and out into an exhaust system. The air velocity at the base of each choice port was maintained at about 15 meters per minute.

Figure 6-1. Schematic drawing of the olfactometer used in laboratory bioassays shown with the white polyethylene foam cylinder and circular fluorescent lamp removed.


Air Input
Activated
Charcoal Filter
Small Mixing
Chamber
Sample
Choice Chamber
Wire Screen Cone
Choice Port
Holding Chamber
To Exhaust

When the olfactometer was ready for use, a removable cylinder of white polyethylene foam (Bernel Foam Products Co., Inc., Buffalo, NY; 40 cm dia, 33 cm ht) was put in place so that the top of the cylinder was flush with the bottom of the mixing chamber. When in place, this cylinder surrounded the choice chambers, blocked all choice chambers from view, and prevented ambient light from striking the choice chambers. A GE Soft White Circleline® Home fluorescent lamp (41 cm dia, 40 watt, General Electric Co., Nela Park, Cleveland, OH) encircled the foam cylinder 20 cm below the mixing chamber. The light which passed through the foam cylinder was dispersed and more evenly illuminated the choice chambers than was the case with previous use of this olfactometer.

On the day before a test, horn flies were sexed under light CO_2 anesthesia and held until the next day in groups of 50 flies in 140 ml plastic specimen containers with screen tops. Until the time of testing the next day, these flies had access to blood meals. At the time of testing flies were 1-2 days old.

For a given test, the 1:1:i mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene in 10-50 µl solutions of hexane was deposited with dispoable capillary pipettes to the center of a Whatman No. 1 filter paper (4.25 cm dia). After evaporation of the hexane, a treated filter paper was placed into 4 consecutive treatment choice chambers by random selection. Filter papers treated with an equivalent volume of hexane were placed in the 4 remaining control choice chambers.

Each test bioassayed the response of 50 male horn flies. Test flies were transferred without anesthesia from the 140 ml plastic specimen container to the bottom of the holding chamber. The number

of flies entering the treatment and control chambers was counted after 15 min. Flies were not retested but were discarded at the end of each test. The olfactometer was disassembled and washed with hot water and detergent between trials when the position of treatment and control choice chambers were altered.

For a given trial, the number of flies entering the 4 treatment choice chambers and the 4 control choice chambers was totaled. The data were transformed ($\sqrt{x + 0.5}$); these transformed values were compared using the paired-t test (Helwig and Council, 1979).

In an additional set of trials 3 doses of the monoolefin mixture were simultaneously tested in the olfactometer during a given test. Two neighboring choice chambers were randomly chosen and received a hexane treated filter paper. The next 2 consecutive choices on both sides of the control choice were each treated with 25 µg or 50 µg of the monoolefin mixture, respectively. The remaining 2 choice chambers were treated with 10 µg per choice chamber. The data were transformed ($\sqrt{x + 0.5}$) before a completely randomized analysis of variance was performed (Helwig and Council, 1979).

Simulated field trials were conducted in outdoor screened cages (1.8 x 1.8 x 2.1 m). Each cage had a concrete floor and a polyethylene cover to exclude rainfall. An electric fly trap (Rid-O-Ray, Flygon Model 2020, Charmglow Products, Bristol, WI) was suspended in the center of each cage. Each trap had been modified so that the light was not functional but the electric grid was still activated. In the center of each trap was a tray to which a filter paper which had been treated with hexane alone or the monoolefin combination could be attached. Attractancy was determined by comparing the catch in traps with filter

papers (Whatman No. 4, 9 cm dia) treated with a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene at 5 mg per filter paper versus traps which contained hexane treated filter papers.

For each of 12 trials, one trap was randomly designated as a control trap and 3 traps were assigned as treatment traps. The appropriately treated filter paper was placed within the trap. Approximately 1000 recently eclosed (1-2 day old) horn flies of about a 50:50 sex ratio were released into the cage. These flies had been counted out in the laboratory about 2 hours previously under brief CO_2 anesthesia and had access to blood meals until released into the outdoor cages.

After the flies were released into the cages at approximately 1600-1700 each test day, the trap grids were activated. After 24 hours, the flies in each trap were collected, sexed, and counted. The data were transformed ($\sqrt{x + 0.5}$) and analyzed by the t-test for 2 independent samples (Helwig and Council, 1979).

Results

The data obtained from olfactometer studies are presented in Tables 6-1 and 6-2. The average response to the treatment choice chambers containing the monoolefin mixture was 24.5%, 28%, and 31.4%, respectively, for the 10 µg, 30 µg, and 50 µg treatment levels; however, only at the 50 µg treatment level was the response of male horn flies to the treated choice chambers significantly greater than was the response to the control choice chambers. In the trials when 3 different doses of the monoolefin mixture were simultaneously presented in different olfactometer choice chambers, there was no significant difference in the number of males attracted to any of the treatment levels or to the control choice ports.

Table 6-1. The response[a] of 1-2 day old male horn flies to doses of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene (1:1:1 mixture) tested in the olfactometer.

Dose[b]	No. Trials[c]	No. Responding to Treatment Choice Chambers	No. Responding to Control Choice Chambers	t-Statistic
10 µg	13	153	206	-1.19 (N.S.)
30 µg	28	373	266	2.03 (N.S.)
50 µg	16	232	161	2.42*

*Paired t value $P < 0.05$.

[a]The data were transformed as $\sqrt{x + 0.5}$ before analysis.

[b]This dose was applied to a filter paper placed in each of 4 consecutive choice chambers.

[c]Fifty male horn flies were tested per trial.

Table 6-2. The response[a] of 1-2 day old male horn flies to doses of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene (1:1:1 mixture) tested simultaneously in the olfactometer.

Dose[b]	No. Responding[c]
control	100
10 µg	106
25 µg	100
50 µg	77

[a]The data were transformed as $\sqrt{x + 0.5}$ before analysis.

[b]This dose was applied to a filter paper placed in each of 2 consecutive choice chambers.

[c]Fifty male horn flies were tested per trial.

In the simulated field trials, less than 3% of the released horn flies were captured in the traps treated with the monoolefin mixture. No significant difference was demonstrated between the catches of treated traps and the control traps at the dose of 5 mg of the monoolefin mixture per trap. When the catches of the traps were considered on the basis of the sex of the flies captured, no significant differences between treated and control traps were demonstrated for the catches of either males or females.

Discussion

These trials demonstrated that male H. irritans were moderately attracted to the combination (1:1:1 mixture) of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene in laboratory olfactometer studies. Mackley (1977) did not demonstrate a significant male response to the total hydrocarbon fraction, the total monoolefin fraction, (Z)-9-pentacosene, or (Z)-9-heptacosene, but did demonstrate a significant male response to (Z)-5-tricosene alone. The combination of monoolefins tested in this study were not bioassayed by Mackley (1977).

In the simulated field trials, the failure of the monoolefin mixture to attract male horn flies may have been due to the low treatment dose of 5 mg per trap particularly since the traps were treated with the monoolefin mixture alone without the addition of baits as was the case with field trials with Fannia sp. (Uebel et al., 1978) and Musca domestica (Carlson et al., 1973; Morgan et al., 1974). The efficacy of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene at higher dosage levels as an attractant both alone and in combination

Table 6-3. Number of H. irritans collected in traps containing 5 mg of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene (1:1:1 mixture) versus control traps.

	Mean No. Collected[a]		
	Treated Traps	Control Traps	t-Statistic
Total	29.3	22.2	0.84 (N.S.)
Males	17.4	13.1	0.70 (N.S.)
Females	12.3	9.3	1.00 (N.S.)

[a] n = 12 trials with 3 treatment traps and 1 control trap per test.

with factors related to the host itself as suggested by the work of Kinzer et al. (1970) remains to be investigated.

APPENDIX A

HORN FLY COURTSHIP DATA

Table A-1. Percent of time spent by individual adult horn flies in different activity categories during 5-minute observation periods.

Pair	Sex	Age (days)	Activity Categories					
			Resting	Cleaning	Walking	Flying	Feeding	Courtship Behavior
1	Female	1	15.1	12.1	67.1	5.7	-	-
	Male	1	17.6	20.2	16.8	45.4	-	-
2	Female	1	13.4	6.5	70.0	10.1	-	-
	Male	1	6.7	13.4	75.2	4.7	-	-
3	Female	1	5.0	3.4	87.0	4.6	-	-
	Male	1	9.6	7.4	77.0	6.0	-	-
4	Female	1	56.7	27.5	39.0	2.4	-	-
		2	57.5	25.6	16.9	-	-	-
		3	90.5	4.6	-	2.0	-	2.9
		4	67.6	19.0	6.7	5.0	-	1.7
	Male	1	17.5	73.8	8.7	-	-	-
		2	6.6	78.8	11.6	3.0	-	-
		3	49.2	25.5	5.3	17.2	-	2.8
		4	29.4	68.7	-	-	-	1.7
5	Female	1	66.9	30.8	2.3	-	-	-
		2	56.5	23.0	14.0	6.4	-	-
		3	25.2	48.1	16.8	5.2	-	4.7
		4	81.0	11.3	3.4	0.2	-	4.7
	Male	1	65.0	35.0	-	-	-	-
		2	71.8	28.2	-	-	-	-
		3	1.3	62.5	9.1	28.4	-	4.7
		4	29.0	27.2	4.0	35.1	-	4.7
6	Female	1	46.7	25.5	26.5	1.3	-	-
		2	57.6	42.4	-	-	-	-
		3	42.6	39.7	2.4	-	-	15.3
		4	44.3	42.9	4.8	8.0	-	-
	Male	1	69.7	30.3	-	-	-	-
		2	35.3	56.2	5.9	2.4	-	-
		3	21.7	44.8	4.5	13.5	-	15.3
		4	6.8	67.1	2.0	1.3	22.8	-

Table A-2. Courtship data for pairs[a] of virgin male and virgin female H. irritans.

Pair No.	Time[b] from start until first strike	Courtship duration	Time from start to beginning of copulation	Copulation duration	Sperm transfer
1	4:00	8:00	12:00	6:18[c]	yes
2	2:00	25:00	27:00	6:01	yes
3	30:02	1:00	30:54	6:26[d]	yes
4	2:49	0:48	3:37	8:21	yes
5	4:18	3:21	7:39	11:23	yes
6	10:50	14:22	25:12	6:08	no
7	18:24	2:02	20:26	9:30	yes
8	11:34	21:16	32:50	3:24	yes
9	7:10	12:38	19:48	6:12	yes
10	0:29	4:01	4:30	5:52	yes
11	16:40	16:45	33:30	5:53	yes
12	10:12	1:29	11:41	6:01	yes
13	1:12	2:11	3:23	5:13	yes
14	11:54	2:26	14:20	7:19	yes
15	0:15	1:45	2:00	4:59	yes
16	5:06	2:59	8:05	5:14	yes
17	6:32	0:12	6:44	4:42	yes
18	1:30	0:59	2:29	4:46	yes
19	2:58	9:08	10:06	4:51	yes

[a]Flies were 4-6 days old and had been reared individually from emergence; 19 pairs were observed.

[b]Time expressed as min:sec.

[c]This pair copulated a second time within 15 min of the first copulation; second copulation duration: 4 min 33 sec.

[d]This pair copulated a second time within 15 min of the first copulation; second copulation duration: 4 min 10 sec.

APPENDIX B

HIERARCHY OF COURTSHIP RESPONSE DATA

Table B-1. Hierarchy of courtship response of 5-7 day old virgin male horn flies[a] to hexane-washed T males treated with varying doses of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene.

Dose per hexane-washed T male (µg)	No. strikes per hour	No. arrested movements per hour	No. of positionings on dorsum	No. copulations
1.25	12	0	0	0
	18	0	0	0
	6	0	0	0
	6	0	0	0
	12	0	0	0
	36	0	0	0
	0	0	0	0
	6	6	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	6	0	0	0
	0	0	0	0
	12	0	0	0
	6	0	0	0
2.50	12	0	0	0
	18	6	0	0
	24	6	0	0
	12	12	0	0
	0	0	0	0
	24	6	0	0
	18	6	0	0
	6	6	0	0
	0	0	0	0
	6	0	0	0
	24	0	0	0
	30	0	0	0
	24	6	0	0
	6	0	0	0
	12	0	0	0
5.00	42	0	0	0
	12	6	0	0
	24	6	0	0
	18	0	0	0
	30	12	0	0
	6	0	0	0
	12	0	0	0
	6	0	0	0
	18	6	0	0
	42	24	0	0

Table B-1. continued

Dose per hexane-washed T male (µg)	No. strikes per hour	No. arrested movements per hour	No. of positionings on dorsum	No. copulations
5.00 cont.	12	0	0	0
	6	0	0	0
	36	0	0	0
	18	0	0	0
	18	0	0	0
10.00	60	18	0	0
	36	12	0	0
	24	6	0	0
	6	0	0	0
	12	6	0	0
	24	0	0	0
	54	18	0	0
	0	0	0	0
	78	54	0	0
	6	6	0	0
	24	6	0	0
	12	0	0	0
	24	0	0	0
	30	12	0	0
	36	12	0	0
20.00	66	6	0	0
	90	24	0	0
	18	0	0	0
	42	6	0	0
	66	30	0	0
	18	12	0	0
	6	0	0	0
	12	0	0	0
	78	18	0	0
	24	0	0	0
	54	24	6	0
	18	6	0	0
	12	6	0	0
	54	12	0	0
	6	0	0	0
40.00	30	24	6	0
	78	12	0	0
	66	18	0	0
	24	6	0	0
	24	0	0	0
	12	0	0	0
	18	0	0	0
	12	0	0	0

Table B-1. continued

Dose per hexane-washed T male (µg)	No. strikes per hour	No. arrested movements per hour	No. of positionings on dorsum	No. copulations
40.00 cont.	30	0	0	0
	42	6	6	0
	24	6	0	0
	36	0	0	0
	36	0	0	0
	0	0	0	0
	12	0	0	0
80.00	48	0	0	0
	66	24	0	0
	66	6	0	0
	12	12	0	0
	48	6	0	0
	60	24	6	0
	36	0	0	0
	42	18	12	0
	36	12	0	0
	24	0	0	0
	24	6	0	0
	30	0	0	0
	48	12	0	0
	48	12	0	0
	30	12	0	0
160.00	96	30	0	0
	72	24	0	0
	42	12	0	0
	12	0	0	0
	66	30	0	0
	66	12	6	0
	6	0	0	0
	0	0	0	0
	54	0	0	0
	0	0	0	0
	18	12	0	0
	42	0	0	0
	84	30	18	0
	36	18	12	0
	0	0	0	0

Table B-1. continued

Dose per hexane-washed T male (µg)	No. strikes per hour	No. arrested movements per hour	No. of positionings on dorsum	No. copulations
Dead female[b] (control)	114	78	18	6
	84	84	0	0
	66	54	0	0
	90	24	6	6
	96	72	0	0
	192	126	18	0
	60	42	6	6
	78	66	12	12
	6	6	6	6
	48	48	12	6
	54	48	6	6
	72	54	0	0
	54	36	0	0
	150	96	12	0
	78	66	12	0
Hexane-washed male (control)	0	0	0	0
	4	0	0	0
	4	0	0	0
	0	0	0	0
	3	0	0	0
	0	0	0	0
	0	0	0	0
	4	0	0	0
	0	0	0	0
	1	0	0	0
	0	0	0	0
	0	0	0	0
	1	0	0	0
	1	0	0	0
	3	0	0	0

[a] In each of 15 trials, the hierarchy of courtship response of 5 virgin males was totaled for each behavior occurring in a 10-minute period; the data were multiplied by 6 to express them as the number of a given behavior per hour.

[b] Females were 5-6 days old.

Table B-2. Number of trials[a] in which specific elements in the hierarchy of courtship response were observed when virgin males were exposed to hexane-washed T males treated with doses of a 1:1:1 mixture of (Z)-5-tricosene, (Z)-9-pentacosene, and (Z)-9-heptacosene.

Dose per hexane-washed T male (μg)	No. trials with strikes	No. trials with arrested movement	No. trials with positioning on dorsum	No. trials with copulations
1.25	10	1	0	0
2.50	13	7	0	0
5.00	15	5	0	0
10.00	14	10	0	0
20.00	15	10	1	0
40.00	15	6	2	0
80.00	15	11	2	0
160.00	15	8	3	0
Dead female (control)	15	15	10	7
Hexane-washed male (control)	8	0	0	0

[a]Each dose was bioassayed in 15, 10-minute trials; during each trial 5, 5-7 day old flies were exposed to the treated T male.

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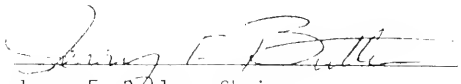
BIOGRAPHICAL SKETCH

Herbert Thomas Bolton was born May 16, 1946, at Camden, New Jersey. After graduating from Haddon Heights High School, Haddon Heights, New Jersey, in 1964, he entered the College of Agriculture and Environmental Science, Rutgers--The State University of New Jersey in New Brunswick, New Jersey. In May 1968, he graduated with the degree of Bachelor of Science in the Preparation for Research Curriculum.

In July 1968, he entered graduate school in the Department of Entomology and Economic Zoology, Rutgers University, as a Thomas J. Headlee Fellow in insect toxicology. In June 1971, his degree of Master of Science in entomology was conferred. In May 1971, he began active duty in the United States Navy on a commission as a Lietuenant Junior Grade previously accepted in 1969. Before entering graduate school in September 1978 for doctoral study in the Department of Entomology and Nematology at the University of Florida, he served three tours of duty as a Navy medical entomologist at Naval installations in the United States and in Okinawa, Japan.

In November 1971, he was married to the former Margie Ellen Gates of San Antonio, Texas. Their two sons are Bryce Daniel and Adam Christopher.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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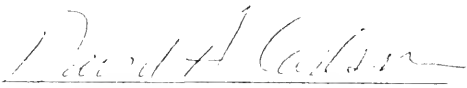
Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



David A. Carlson
Associate Professor of Entomology
and Nematology

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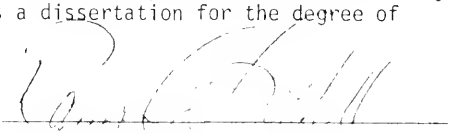

Ramon C. Littell
Associate Professor of Statistics

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James L. Nation
Professor of Entomology and
Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard H. Roberts
Professor of Entomology
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philogophy.

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